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CORNEAL DEVELOPMENT

I. CORNEAL TRANSPARENCY [1]

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New Haven 11, Connecticut

SIX FIGURES

INTRODUCTION

It is held that the vertebrate cornea is transparent from the time it first appears until it matures, (Mann, '50). A casual examination of the chick cornea during its development reveals that this is not so. The cornea, like other embryonic tissues, is, in fact, highly translucent at very early stages; but it becomes progressively more opaque as development proceeds, until a reversal occurs and the cornea acquires the adult transparency (van den Hooff, '51). Failure to observe intermediate stages may have led to the earlier misconceptions.

The fact that the cornea achieves adult transparency during a restricted period of development invites both descriptive and experimental investigation. For, although much work has been done with adult corneas in an attempt to uncover the factors underlying corneal transparency, (eg. Hart and Chandler, '48a, '48b; Davson, '55; Cogan and Kinsey, '42, etc.) the study of adult tissues has limitations. Thus, in the fully developed cornea a large complex of factors and conditions coexist in time and are difficult to separate experimentally; while in the developing cornea factors come into operation, and conditions into being, in an orderly temporal sequence. A knowledge

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of the order in which events occur during corneal development, or detection of their concurrance, would be of great value in establishing the physical basis for corneal transparency.

Studies of adult cornea have implicated a number of factors affecting corneal transparency. Of these, corneal hydration, stromal mucopolysaccharides and the degree of packing of the stromal collagen are most often mentioned. The present study describes the order of appearance of these factors and conditions during the development of corneal transparency in the chick embryo.

Both quantitative and qualitative methods were used to determine the time course of development of transparency, the change in corneal water content as transparency develops, the localization of stromal metachromatic mucopolysaccharides at different ages, and the developmental changes in stromal collagen. The results, of intrinsic interest, contribute, in addition, to the background of information available for experimental studies of the developmental physiology of the cornea.

MATERIALS AND METHODS

The animals used were chick embryos from eggs incubated at 37.5°C in a forced draft incubator. All ages are given as days following the onset of incubation.

Two related optical properties of the cornea, transparency and light transmission, were investigated. Corneal transparency was demonstrated at different ages by a photographic procedure. Corneas of each age were removed quickly from the embryonic eye and laid, convex side up, over a copper grid (180 perforations per cm) in the bottom of a flat glass dish filled with a 0.05 M aqueous solution of NaCl. Using an optical system which magnified six times, the grid was photographed through the cornea under standardized conditions of transmitted lighting and exposure. The exposed films were developed and printed under uniform conditions. This method was used solely to illustrate qualitative changes in transparency during development.

To measure the percent of incident light transmitted by corneas of different ages, a photometric procedure was employed. The corneas were isolated and immersed as described above except that the copper grid was omitted. The glass dish with its contained cornea was placed in a photometer in such a way that a narrow, columnated beam of white light passed through the center of the cornea before striking the photocell. Since the photometer had previously been balanced at a 100% reading with the fluid filled dish alone in the light path, readings could be read directly as percent light transmission by the cornea.

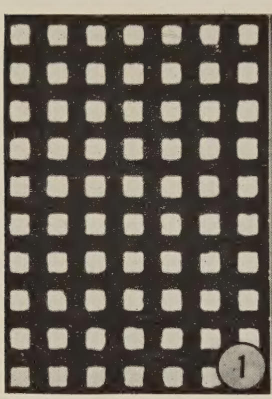
To determine changes in corneal water content, measurements of corneal wet weight and dry weight were made. The ease with which the cornea is isolated and the accuracy of corneal weight determinations were increased by the nature of the corneal limbus of the chick during the period under study. A thin band of collagen surrounds the chick cornea and separates it from the row of peri-corneal cartilages. With the use of watchmakers' forceps the cornea separates cleanly from the eye along this collagenous band. Corneas thus isolated were blotted on filter paper and weighed immediately to the nearest 0.01 mg in aluminum tares, on a torsion spring balance (25 mg capacity). Subsequently the corneas, still in their tares, were dried at 110°C for one hour (a procedure which reduced the corneas of all the ages studied to constant weight) and reweighed. These measurements were made on ten corneas on each day between the ninth and twenty-fourth inclusive. From the mean wet weights and dry weights so obtained, corneal water content was calculated as mg of water per mg of dry weight.

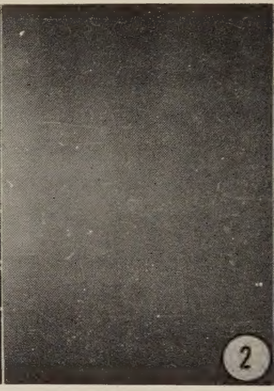
To localize corneal metachromatic mucopolysaccharide in time and space toluidine blue staining was used. Eyes were fixed in Rossman's fluid at daily intervals from the tenth to the twenty-fourth days. These eyes were embedded in paraffin, sectioned at 6 μ, affixed to slides, deparaffinized in xylene and hydrated through a series of alcohol solutions. Sections so prepared were stained in a 0.035% aqueous solution of toluidine-blue-0 at pH 3.5-4.0 and then dehydrated and mounted in balsam.

RESULTS

It was necessary at the outset of this study to determine accurately the time course of development of corneal transparency. This information permitted a more directed search for correlated events.

Changes in corneal transparency during development. The chick cornea is opalescent prior to the fourteenth day, but thereafter becomes more transparent until, by the nineteenth day, it assumes the glasslike transparency characteristic of the adult cornea. The photographs taken to demonstrate the changes show a scarcely discernible grid image prior to the fourteenth day. Between the fourteenth and the nineteenth day the grid images become increasingly clear (figs. 1-3) due to an increase in the sharpness of the image and to an increase in the contrast between the images of the grid bars and the grid interstices. The latter observation suggested that in addition to an increase in transparency, per se, there is also a





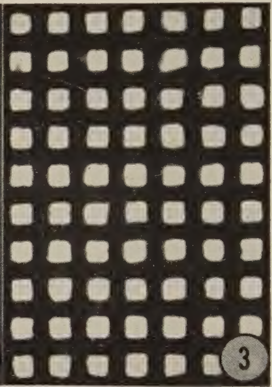


Fig. 1 Copper grid photographed by transmitted light without an overlying cornea.

Fig. 2 Copper grid photographed by transmitted light through a 13 day chick embryo cornea.

Fig. 3 Copper grid photographed by transmitted light through a 20 day chick embryo cornea.

related increase in the amount of light transmitted by the cornea.

Changes in corneal light transmission during development. To measure the transmission of light by the developing cornea, the photometric technique described above was employed. Measurements of per cent transmission were made on 10 corneas on each day between the tenth and twenty-first days inclusive (fig. 4). Before the fourteenth day the chick cornea transmits about 40% of incident light. After the fourteenth day the amount of light transmitted rises sharply until the nineteenth day, when the adult level (about 96% under our conditions of measurement) is achieved. Once the increase has begun it proceeds without change in rate until it levels off at 19 days. Changes in both transparency and transmission are begun and are completed rather abruptly. We are thus sup-

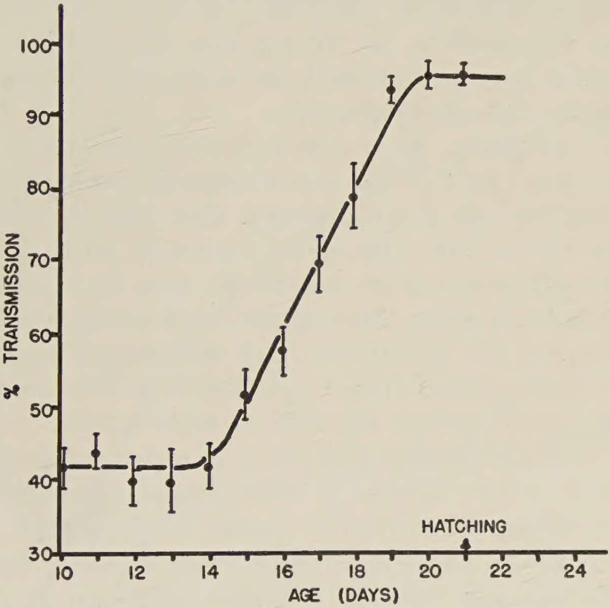


Fig. 4 The percent of incident light transmitted by the chick embryo cornea at different ages. Each point represents the mean of measurements on 10 corneas. The vertical lines indicate standard deviation.

plied with well defined times in development when changes can be sought that are correlated with the development of a transparent cornea.

Changes in corneal water content during development. The importance of the amount of water in the cornea in relation to transparency has been emphasized by many investigators dealing with the adult cornea. It seems, therefore, desirable to know something of the changes in the relative water content of the cornea during the period in development when it is becoming transparent. To obtain this information, wet and dry weights were determined for each day during the last half of embryonic development.

The dry weight of the cornea increases steadily as development proceeds (fig. 5). The wet weight, by contrast, increases rapidly until about the fourteenth day, and thereafter at a markedly slower rate (fig. 5). Correlated with this change is the fact that prior to the fourteenth day, the cornea is relatively thick, being more plano-convex than concavo-convex, and that following the fourteenth day the cornea appears less swollen and its epithelial and endothelial borders become progressively more parallel throughout their extent.

These findings suggest that there is a progressive, relative dehydration of the cornea as development proceeds. This is confirmed when corneal water content is calculated at different ages (fig. 6). There is a relative loss of water until about the nineteenth day, when it becomes stabilized at the adult value. Relative water loss is most rapid between the fourteenth and nineteenth days.

Corneal metachromatic mucopolysaccharides. The search for other events correlating in time with the development of corneal transparency led to an investigation of the appearance during development of metachromatic, stromal mucopolysaccharides. Sections of corneas from embryos less than fourteen days old which are stained with toluidine blue reveal no metachromatic material in the corneal stroma. After the fourteenth day metachromasia is detected first at the endothelial border of the stroma just inside Descemet's membrane. As development proceeds the metachromatic zone intensifies and spreads toward the epithelial border of the stroma. The entire stroma is metachromatic by the eighteenth or nineteenth day.

Coextensive with the spread of metachromatic material tightly packed, linearly oriented bundles or plates of collagenous fibers are laid down in successive portions of the stroma. This is in contrast to the sparse and disoriented condition of collagen fibers prior to the advent of metachromasia.

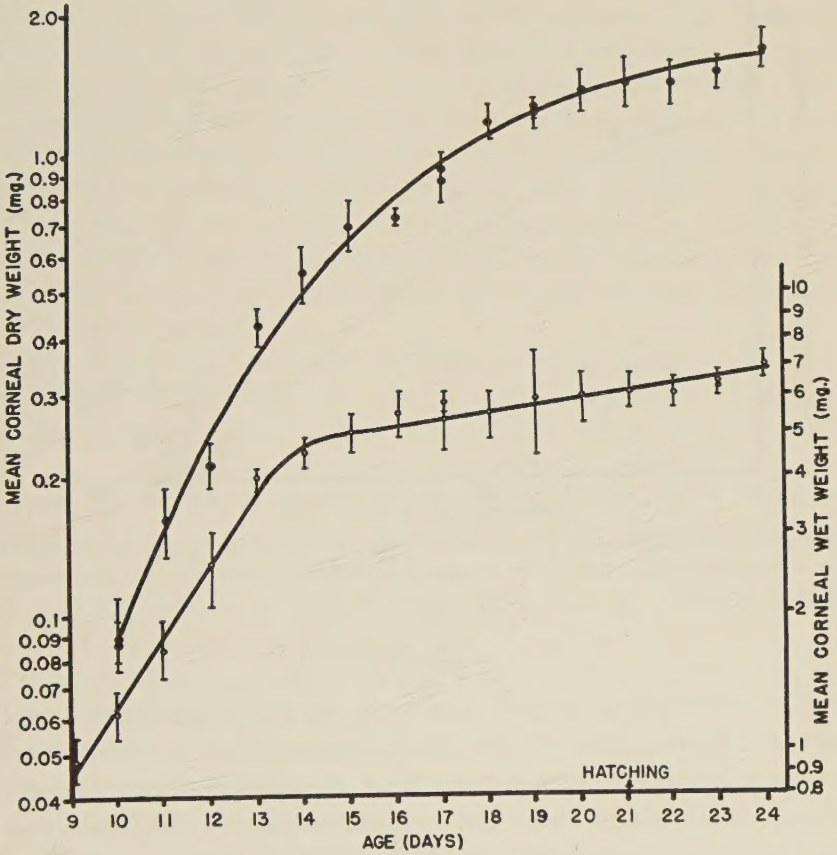


Fig. 5 Semi-log plots of corneal dry weight (upper curve), and corneal wet weight (lower curve) as functions of age. Each point represents the mean of measurements on 10 corneas. The vertical lines indicate standard deviation.

A qualitative observation correlates well with the above facts. Before the fourteenth day the cornea is soft and is easily torn apart. On the fourteenth day it begins to toughen and becomes more difficult to tear with forceps as development proceeds and more tightly packed collagen is laid down.

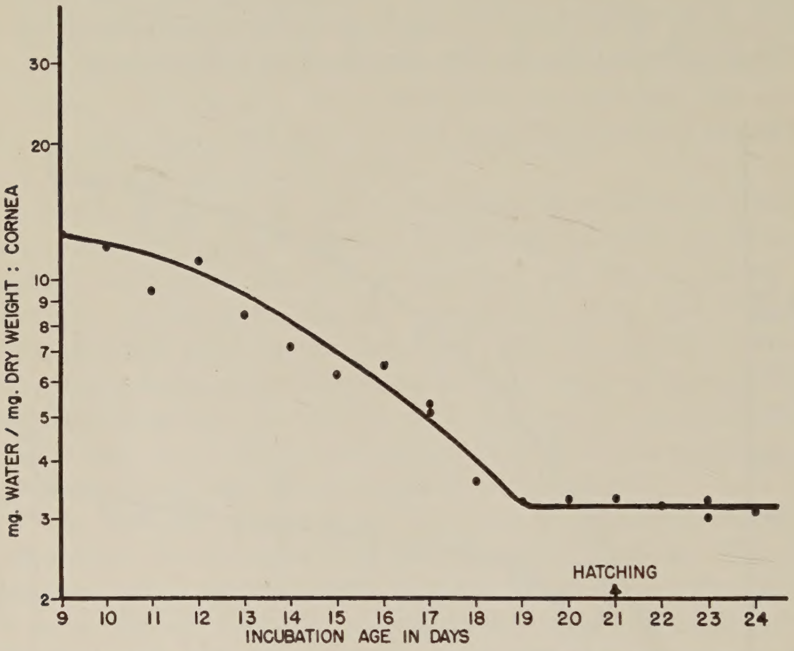


Fig. 6 Semi-log plot of the amount of water per unit dry weight of the cornea as a function of age. Each value is calculated from the wet weight and dry weight data in figure 5.

DISCUSSION

The sequence of events that emerges from this descriptive study affords clues of the relative roles played by each of several factors in the development of corneal transparency. The establishment of causal sequences must await the more critical evidence of experimental studies of the developing cornea. In the meantime, the data presented here not only suggest the experiments which must be done, but indicate the times at which important changes occur in the developing cornea, and at which experimental interference would prove more informative.

Prior to the fourteenth day the cornea is relatively thick, opaque, contains relatively disoriented collagen, holds a large amount of water relative to dry weight and contains no detectable metachromatic material. The cornea shows an increase in light transmission and in transparency beginning on the fourteenth day. At the same time a group of associated changes are initiated. Metachromatic material appears in the stroma. Densely packed stromal collagen is seen for the first time. Corneal water loss is accelerated. The cornea becomes thinner. As the cornea increases in transparency between the fourteenth and nineteenth days there is a progressive increase in the absolute and relative stromal volume occupied by metachromatic material and dense collagen, and a continued loss of water relative to dry weight. On the eighteenth or nineteenth days metachromatic material and dense collagen fill the stroma. By the nineteenth day the water content levels off at the adult value.

Simultaneously with the publication of an abstract of these findings on the chick (Coulombre '56), Smelser and Ozanics ('56) published an abstract of a similar study of the rabbit cornea. In all major respects the results reported in both abtracts are similar. Therefore the concurrence of a relative loss of water, a deposition of metachromatic material, the appearance of closely packed collagen and the development of transparency may also be the general course of events in mammals during the development of the cornea.

The general increase in transparency between the fourteenth day and hatching reported by van den Hooff ('51) has been confirmed in the present study. The transient decrease in transparency at nineteen days which he describes did not occur under our conditions of measurement. This discrepancy in results is not yet explained.

The cornea begins to show a relative loss of water, without a concomittant increase in transparency, before the pivotal fourteenth day. This suggests that, while an appropriate level of corneal dehydration is a necessary condition for transparency, it is not a sufficient condition. The first appearance of metachromasia and packed collagen at the same time that corneal transparency begins to increase, suggests that these events are of more central importance in the development of corneal transparency. The facts that packed collagen and metachromatic mucopolysaccharide appear at the same time and in the same place, and spread at the same rate through the thickness of the cornea, suggest an intimate relationship between metachromatic mucopolysaccharide and the oriented packing of the collagen fibers.

Electron micrographs of chick embryonic cornea reveal stromal collagen at very young ages (personal communication from Dr. Marie Jakus). It is still an open question whether the changes in stromal collagen which begin on the fourteenth day represent an increase in the rate of collagen synthesis or simply a packing of preexisting collagen plus new collagen being laid down at an unchanged rate. If the former alternative holds one would expect an abrupt change in the rate of increase of corneal dry weight. Since no such abrupt change occurs (fig. 5) we are inclined to accept tentatively the latter alternative.

SUMMARY

1. In the chick, corneal transparency and light transmission begin to increase on the fourteenth day of incubation, and achieve adult levels by the nineteenth day.

2. The relative water content of the cornea decreases from a level of well over 90% of wet weight at 9 days to the adult level of 76% at nineteen days. The period of most rapid decrease lies between the fourteenth and nineteenth days.

3. Metachromatic mucopolysaccharide appears in the stroma for the first time on the fourteenth day, just inside Decemet's membrane. It spreads throughout the stroma during the ensuing 4 or 5 days.

4. Densely packed stromal collagen appears at the same time and in the same place as the metachromatic material, and spreads through the stroma at the same rate.

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A COMPARATIVE STUDY OF SOME EXCITABILITY PROPERTIES OF THE GIANT AXONS OF THE VENTRAL NERVE CORD OF THE LOBSTER, INCLUDING THE RECOVERY OF EXCITABILITY FOLLOWING AN IMPULSE

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TEN FIGURES

INTRODUCTION

For the past several years a study of excitability and conduction in single crustacean nerve fibers has been carried out which, for the most part, has been limited to the motor axons of the walking limbs of the lobster and the chelae (claws) in fresh water crayfish (Wright and Coleman, '54). A few experiments have been reported in which the effect of potassium ions on the excitability of giant fibers isolated from the ventral nerve cord (Johnson, '24; Wiersma, '47) is described (Wright et al., '55). These fibers as well as certain axons found in the chelae of the lobster are much larger than those found in the walking limb and hence a desirable preparation especially for intracellular electrode studies of nerve (Tobias and Bryant, '55). It is therefore important to extend the studies on excitability and conduction of single nerve fibers to include these unusually large motor axons and compare the properties of these fibers with the properties of other smaller ones. Because it is believed that the preparation used has several advantages over another preparation of the same fiber (Tobias and Bryant, '55) the present work also includes a detailed description of the procedure for the isolation of these giant axons from the esophageal commissure region of the ventral nerve cord. This technique is not new, having been used for some time in studies of the synapse in the crayfish (Wiersma, '47).

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METHODS

At the anterior end of the ventral nerve cord of the lobster there are two large fused ganglia. At the very end is the supra-esophageal ganglion or brain of the animal and some 4–5 cm from this terminal brain ganglion is the second large fused ganglion, the sub-esophageal ganglion. The ventral nerve cord connecting these two large ganglia is divided into two connectives, the esophageal commissures, which circumvent the esophagus.

To expose these large nerve bundles, the commissures, the carapace in the thoracic area is cut away and the underlying viscera including the green glands carefully removed. The head end of the animal is isolated just below the sub-esophageal ganglion region and the carapace trimmed away leaving only a narrow portion of shell along the ventral side to serve as a support of the commissures and surrounding tissue. The anterior end of the shell is fixed in a clamp and the tissue immersed in Cole's solution (Cole, '41) or sea water. The preparation now appears, under the low power dissecting microscope, as shown in the photomicrograph in figure 1a. The two commissures are ready for dissection. Each is surrounded by a thick connective tissue envelope. This sheath is easily pierced with a sharp needle probe, and once an opening is made it may be enlarged with a single stroke of the needle forward and back into a longitudinal slit in the sheath extending the entire length of the commissure. It is advisable to make this slit in the side rather than the upper surface of the sheath to avoid damaging the giant fibers which lie along the top or dorsal surface of the commissure just below the sheath. The slit in the sheath is carefully pulled apart with needle and forceps and the nerve bundles gently teased out into the solution. The giant fibers because of their size are immediately visible along the upper surface of the nerve bundle. The connective tissue around the fibers is extremely soft which makes it a relatively simple matter to isolate one giant fiber and, while holding it away from the sheath and nerve with a wire hook, cut away the nerve. This leaves a single fiber preparation as shown in the right commissure of figure 1. Then the fiber, with a small tuft of nerve tissue at either end, is freed from the commissure and mounted on three electrodes in the form of a V as described elsewhere (Wright et al., '55) and the excitability and electrical properties studied with the results given below. These properties have been compared to those of the large motor axons of the chelae and walking limbs.

One advantage of this preparation over others described (Wiersma, '47; Tobias and Bryant, '55) is in the length of fiber obtainable. Up to four cms may be isolated from large animals. Another is the relative ease with which one may procure an extremely clean nerve fiber which is essential for single fiber studies (Marmont, '41). Twenty giant fiber preparations have been used.

RESULTS

The conducted action potential of two fibers of the ventral nerve cord is shown in figure 2a. The medial giant, 125 micra in diameter, contributed the larger spike, the other response coming from a fiber 70 micra in diameter of unknown function but included in the preparation for comparison. The velocity of conduction of the medial giant spike was 18 M/sec, of the smaller fiber 7 M/sec. The conduction velocity of all giant fibers used ranged between 18 and 20 M/sec. The action potential spikes of this double fiber preparation were about 60 and 30 mv in magnitude and slightly less than 1 msec in duration, respectively. The all or none response appeared no different than the responses of other lobster nerve fibers, except that repetitive firing has never been obtained, even with strong D.C. stimulation, from the large medial giant fiber.

In two cases oscillatory responses were recorded from smaller giant axons, perhaps lateral giants, and both of these preparations were dissected from the same animal. The results have not been duplicated in any other preparations.

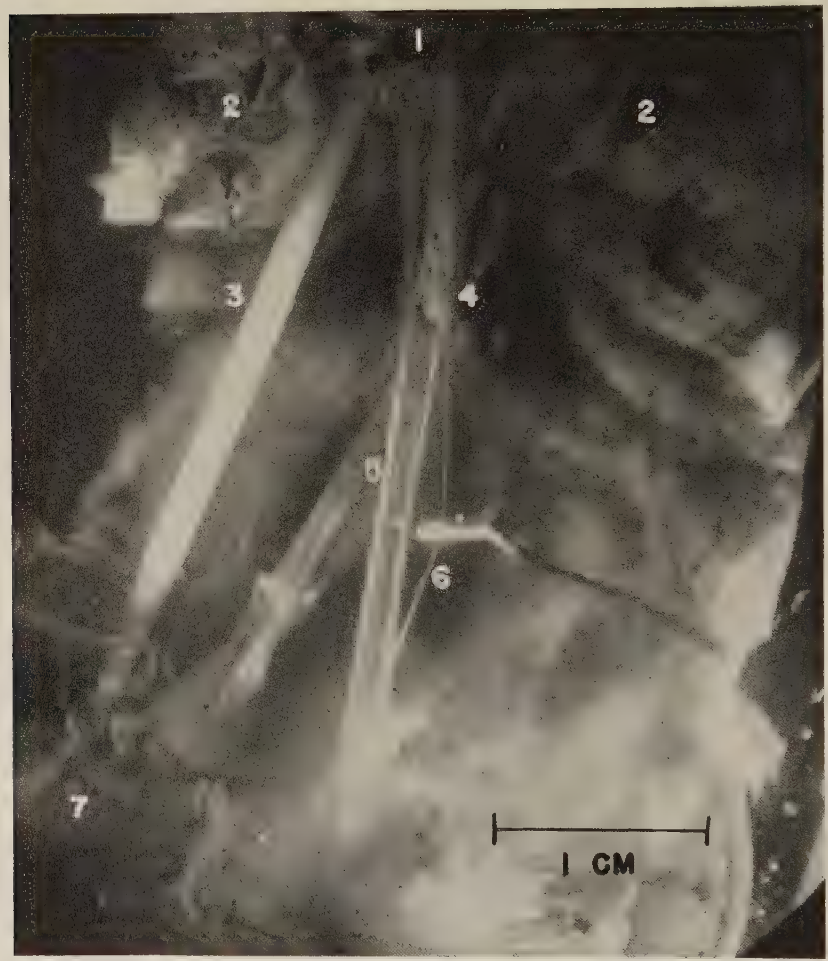


Fig. 1 Microphotograph under low power of single giant axon preparation in the esophageal commissure of the lobster. 1. brain ganglion, 2. eye area, 3. left commissure intact, 4. nerve stump in right commissure, 5. sheath of right commissure, 6. giant axon, (Ca 100 μ diameter), hooked over wire probe, 7. esophagus tissue.

The strength duration curves of the two fibers, figure 2a, are shown in figure 2b. In this instance the threshold of the larger fiber was less than the threshold of the smaller fiber but the curve was sharper and the chronaxie value (0.7 msec compared to 1.1 msec) was less. The excitability rate constant k was 1.0×10^3 sec $^{-1}$ for the giant fiber, a value about the same as found for fast closer fibers or other non-oscillatory ones (Wright and Coleman, '54).

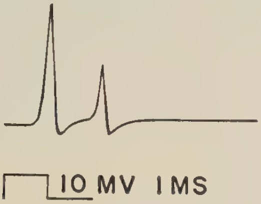


Fig. 2a Action potentials of medial giant, 125 μ diameter, large spike and another unidentified 70 μ diameter fiber isolated from the commissure.

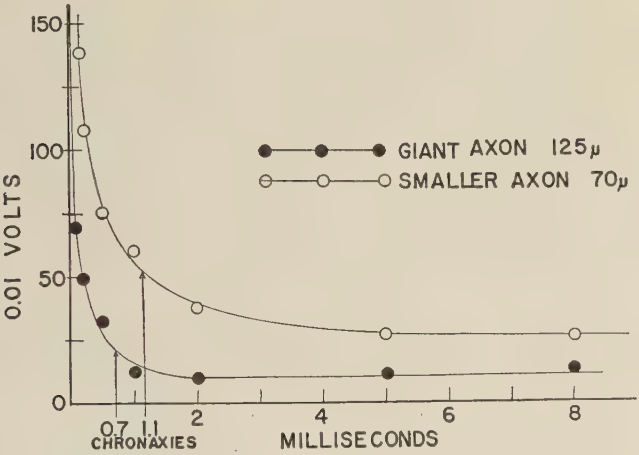


Fig. 2b Strength-duration curves of these two ventral nerve cord fibers.

The electrical responses to threshold and subthreshold D.C. stimulation recorded directly at the cathode from another 120 micra diameter giant axon are illustrated in figure 3a.

The upper two traces were obtained right after dissection and the lower two traces, 1½ hours later. The only significant difference is in the increased duration of the local response, lower trace, with subthreshold stimulation after 1½ hours, and the increased latency of the spike at that time with threshold stimulation. The strength-duration curves of this fiber, also obtained right after dissection and 1½ hours later, are shown in figure 3b. The curvature became less acute and approached a slightly reduced rheobase value after 1½ hours. Excitability curves obtained by the dual shock method are shown in figure 4b. The reduction in threshold at the onset of the subthreshold conditioning shock was brief and increased rapidly after only two milliseconds in the freshly dissected fiber. However, after 1½ hours the threshold value remained very low for 4 msec and then increased very slowly and only slightly during the next 10 msec.

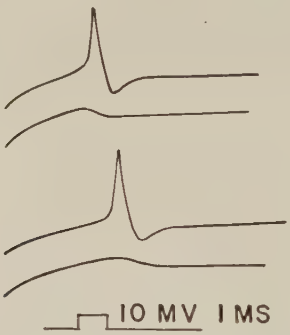


Fig. 3a Action potentials from 120 μ diameter medial giant elicited by threshold and subthreshold stimulation (upper and lower traces) right after dissection upper two traces, and 1½ hours later, lower two traces.

The recovery of excitability as measured by the short duration shock voltage required to stimulate at various time intervals after the discharge of an impulse is plotted in figure 4a. The curves obtained immediately after dissection (open circles) and 1½ hours later (solid circles) differed in one important aspect. The data taken after 1½ hours showed the development of a super-normal or hyper-excitable phase after the recovery process as illustrated by the "dip" or overshoot of the threshold value beyond normal during the first 4 msec. The vertical dashed lines indicated also that the refractory period had reduced slightly during this "rest period." Curves containing a hyper-excitable or super-normal "dip" were always obtained from the highly excitable oscillatory fiber type (Hodgkin, '48) as shown below.

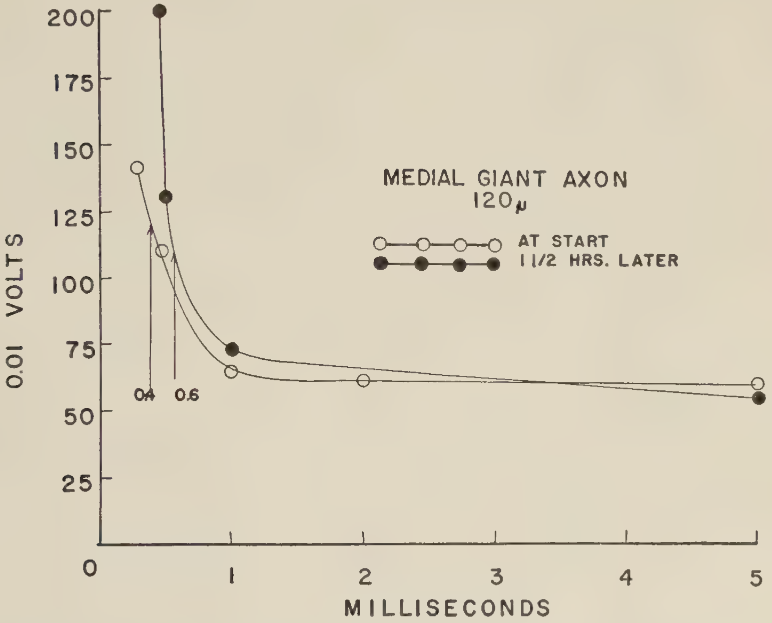
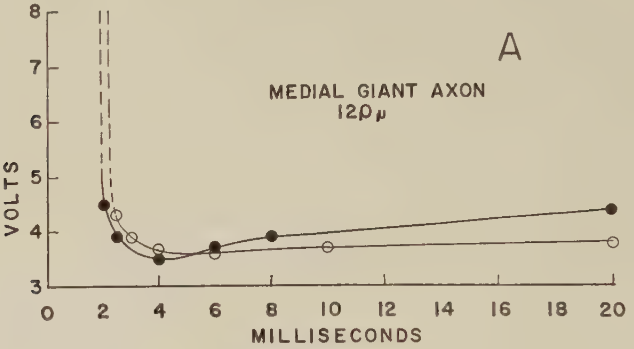


Fig. 3b Strength-duration curve of this axon freshly dissected (open circles) and 1½ hours later (solid circles). Chronaxie values are indicated.

It is evident from the data that during the hour and one-half in Cole's solution the giant fiber became more excitable and less accommodating as shown by the change in curvature of the strength-duration curves and the dual shock excitability curves. It is concluded then that the lengthened duration of the local response (and latency) and the change in curvature of the excitability recovery curve to include a super-normal phase are also to be associated with an increased excitability and decreased accommodation.

The strength-duration curves of the fast closer fiber, 95 micra diameter, and opener fiber, 80 micra in diameter, isolated from the chela are compared with those from the same fibers of the walking leg in figure 5a and b. The data obtained from the chela fast closer fiber, almost as large and in some cases larger than the giant fibers of the ventral nerve cord, is much different than that obtained from the opener fiber. The chronaxie value was 0.4 msec for the fast closer, 0.95



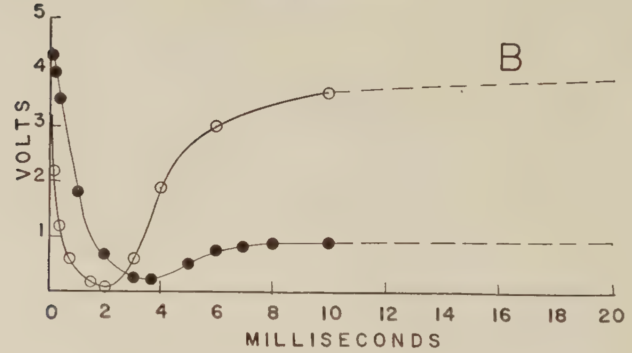


Fig. 4a Curves showing recovery of excitability following an impulse in the 120 diameter medial giant axon freshly dissected (open circles) and 1½ hours later (solid circles).

Fig. 4b Excitability curves of the axon obtained by the dual shock method right after dissection (open circles) and 1½ hours later (closed circles).

msec for the opener and the rheobase value of the fast closer was about three times greater than the opener. The excitability data from the two fibers with the same function in the walking leg were more nearly alike, the curves almost overlapping in this preparation.

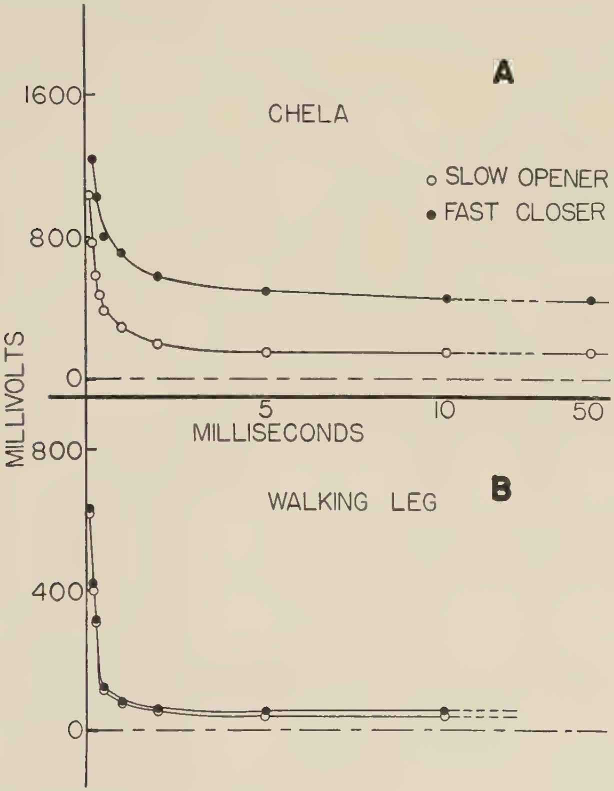


Fig. 5a Strength-duration curves of fast closer fiber, 95 μ diameter (solid circles) and opener fiber, 80 μ diameter (open circles), from the chela.

Fig. 5b Strength-duration curves of the fast closer fiber, 70 μ diameter (solid circles) and opener fiber, 60 μ diameter (open circles) from the walking leg.

The effect of lowering the temperature on the strength-duration data of the large fast closer from the chela is shown in figure 6. The rheobase value is markedly reduced and at the same time the chronaxie value is increased. Thus the curve is shifted downward and to the right, relative to the axes (Wright, '57).

Excitability and recovery curves of the fast closer and opener of the chela are compared in figure 7. The fast closer data was obtained at two different temperatures. There was virtually no significant hyper-excitable phase during recovery in the fast closer at either 23°C (open circles) or 6°C (solid circles), but there was a very pronounced overshoot of the threshold value during the recovery of the opener fiber (X's, dashed line curve). The numbers on the right of the ordinate indicate the threshold values of this curve. Cooling caused the recovery curve to be shifted to the right, obviously because of the lengthening of the refractory period at low temperatures. It is also slightly raised relative to the axes due to the increase in threshold of short duration shocks (Wright, 57) by cold.

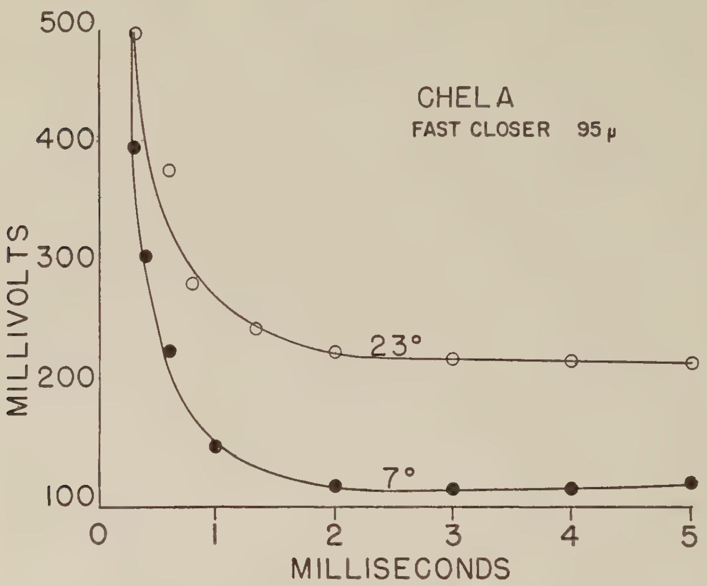


Fig. 6 Strength-duration curves of the fast closer fiber, 95 μ diameter, of the chela at 23°C (open circles) and 7°C (solid circles).

The excitability recovery curves of an opener fiber of the walking leg obtained immediately after dissection (curve A) and 1½ hours later (curve B) are shown in figure 8. There was a marked change in the shape of the curves during this "resting period." The fiber was non-oscillatory at first and it will be noted there was no overshoot or super-normal phase in the first recovery curve. Later on the "dip" became very pronounced and at the same time the response of the fiber to D.C. stimulation became oscillatory. The effect of cooling on the excitability recovery curve of another opener fiber of the walking leg is shown in figure 9. The effect is even more pronounced than in the case of the fast closer, figure 7. The refractory period was increased from one to sixteen ms and the threshold for short duration shocks almost tripled by the cold temperature.

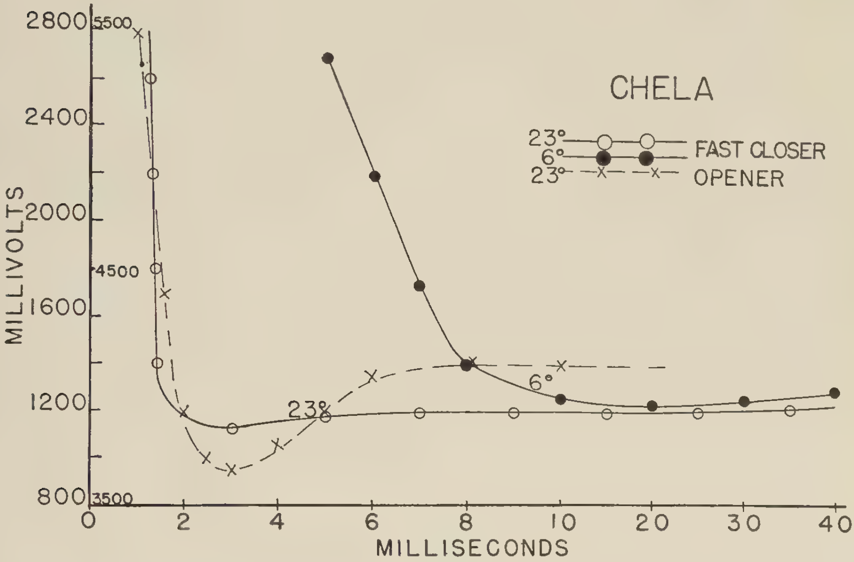


Fig. 7 Excitability recovery curves of the fast closer, 95 μ diameter, of the chela at 23°C (open circles) and at 6°C (solid circles). The excitability recovery curve of the opener, 80 μ diameter, at 23°C is included (dashed line, X's). The smaller numbers on the right of the ordinate are for this latter curve data.

Finally, the difference between the excitability recovery curves of the two distinct fiber types, the fast closer and slow closer of the walking limb, is clearly shown in figure 10. There was only the slightest indication of a hyper-excitable or supernormal phase during the recovery of excitability in the larger and less oscillatory responding fast closer. The recovery curve of the smaller highly oscillatory responding slow closer fiber had a very pronounced hyper-excitable "dip."

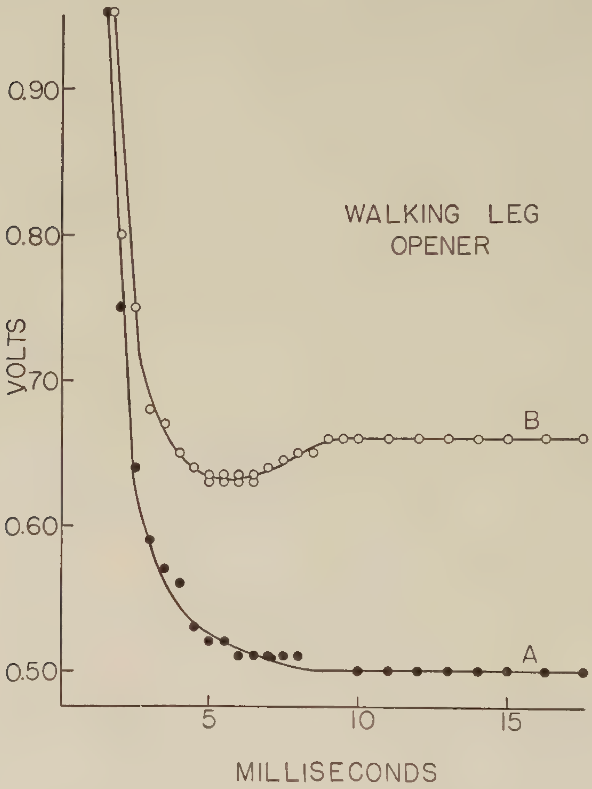


Fig. 8 Excitability recovery curves of an opener fiber of the walking leg, 55 μ diameter, immediately after dissection (A, solid circles) and 1½ hours later (B, open circles).

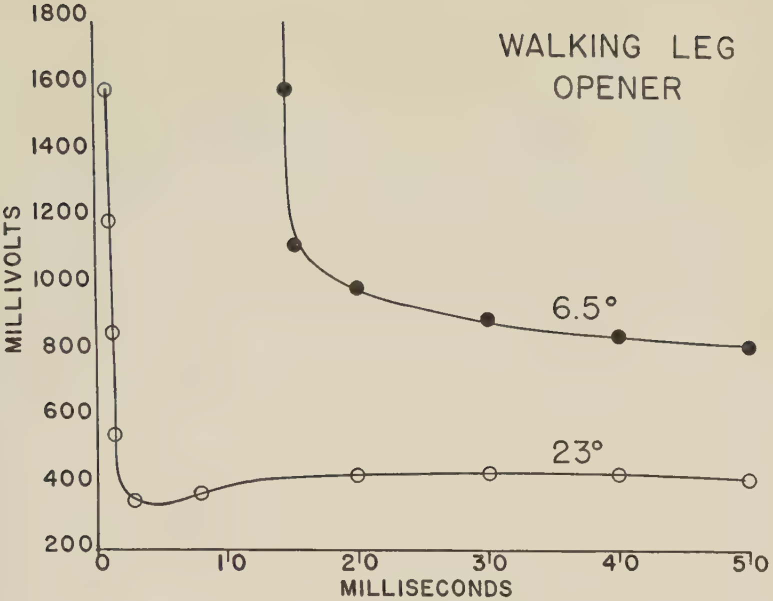


Fig. 9 Excitability recovery curves of the opener fiber of figure 8, two hours after dissection at 23°C (open circles) and at 6.5°C (solid circles).

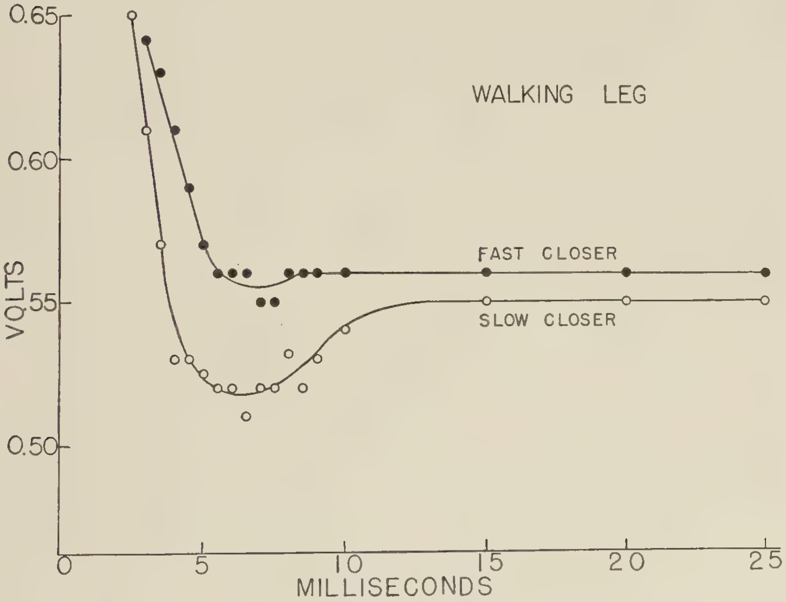


Fig. 10 Excitability recovery curves or the fast closer fiber (solid circles) and slow closer fibers (open circles) of the walking leg of the lobster.

DISCUSSION

The above comparison of excitability properties of the giant motor axons of the ventral nerve cord, chela, and walking limb of the lobster has shown that the ventral nerve cord giants are non-oscillatory and should be included in group 3 of Hodgkin's classification for non-myelinated axons (Hodgkin, '48). The strength-duration curves of the more excitable fibers are less acute in curvature than those of the highly damped giant fibers as might be expected (Wright and Coleman, '54). Furthermore, the more excitable fibers, especially the highly oscillatory responding ones, enter into a transient super-normal phase during recovery following an impulse discharge as shown by a pronounced "dip" in the recovery curves of these fibers. This hyper-excitable phase was insignificantly small in most giant axon preparations if it appeared at all.

It is interesting to consider just one aspect of this super-normal phase of recovery. If during recovery from the initial discharge elicited by an applied D.C. current of threshold value the excitability of the fiber enters a super-normal phase, there will be a great tendency for the fiber to discharge again during this hyper-excitable period. The repetitive firing rate would then be limited, in a sense, to the duration of this super-normal phase. Thus an opener fiber as an example of the extremely oscillatory responding type should be able to fire repetitively over a large frequency range, whereas a fast closer fiber might be expected to respond repetitively but only within a small relatively high frequency range. It has been observed (Wright and Coleman, '54) that the rate of firing of an opener fiber may be extremely rapid at the beginning of a burst of spikes or relatively slow, especially towards the end of a volley, while a fast closer fiber often responds with a short burst of spikes at a high frequency. It is not known what the cause of the super-normal phase of the excitability recovery is. The correlation of this with ionic and voltage variations in the membrane has yet to be determined.

In table 1 are given the range in values of the various properties of all the lobster axons investigated to date. The giant axon of the ventral nerve cord is usually the largest, the opener of the walking leg the smallest of those included. The velocity of conduction, greatest in the giant axon, is certainly not related directly to axon diameter, but may be correlated in some way with the k value, also largest in the giant axon. Fibers with large k values are usually extremely big fibers, or perhaps damaged ones. In our original studies (Wright and Coleman, '54) many k values above 2.5 were obtained, but now after improvements in dissecting technique such a value is to be associated with an abnormal fiber, either damaged or otherwise in poor condition. The opener fiber, not always the smallest one, is invariably the most excitable as indicated by the very pronounced an long enduring super-normal phase during recovery from an impulse, and the highly oscillatory nature of the discharge. Oscillatory responses have not been recorded either from the nerve cord giant axon or the large fast closer of the chela. There is little or no super-normal phase in the recovery curves of these very large fibers.

TABLE 1

Properties of lobster axons

	CORD GIANT	CHELA FAST CLOSER	OPENER	LEG FAST CLOSER	OPEN
Diameter	90–180 μ	80–125	70–90	70–100	40–80
Velocity	18–20 M/sec	18–20	14–18	12–18	7–13
k value	1.0–2.0 × 10^3/sec	1.0–1.7	0.75–1.0	0.75–1.5	0.35–0.75
Supernormal phase duration	0–2 msecs	0–2	2–8	0–5	5–10
Oscillatory responses	none	none	++	+	++++

SUMMARY

A preparation of the giant axons in the esophageal commissure region of the ventral nerve cord of the lobster has been described. The excitation and some electrical properties of these axons have been described and compared to other large motor axons of the lobster.

The electrical response of the ventral nerve cord giant axon is the same as the response of other large crustacean fibers in most respects. The giant axons of the cord are non-oscillatory, have small chronaxie values, large k values, considerable accommodations and may be classified with the large fast closer axons of the chela of the lobster and of the fresh water crayfish, group 3 (Hodgkin, '48).

The fast closer of the walking leg of the lobster possesses excitability properties more nearly the same as those of the repetitive type firing fibers such as the opener or slow closer fibers.

The giant axon and large fast closer of the chela recover excitability usually without a super-normal phase or only a very slight one whereas the smaller fibers recover excitability through a long, large super-normal phase. This hyper-excitable state during recovery seems to be associated with the ability to fire repetitive discharges and is significantly absent in the non-oscillatory responding axon.

Some implications of these results are briefly discussed.

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THE EFFECT OF LOW TEMPERATURES ON SINGLE CRUSTACEAN MOTOR NERVE FIBERS

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SEVENTEEN FIGURES

INTRODUCTION

It is a rather extraordinary fact that nerves from different animals, indeed sections of nerves from the same animal, are adapted to function normally within a particular temperature range specific to the nerve or nerve section. Mammalian nerves, for example, function optimally at 35–37°C (Chatfield et al., '48) a temperature at which invertebrate nerve conduction fails completely (Hodgkin and Katz, '49) and frog nerve function is markedly impaired (Gasser, 31). On the other hand, frog and invertebrate nerve which functions normally at 20–25°C remains excitable with nearly freezing temperatures; whereas mammalian nerve function generally fails at 12–15°C (Chatfield et al., '48; Hodgkin and Katz, '49; Gasser, '31) with the exception of nerves of certain hibernating species which may conduct impulses at near freezing temperatures just as well as frog or invertebrate nerves. Indeed, it has been found in the Herring Gull peroneal nerve that action potentials are elicitable at 3°C in a small nerve section which is normally subjected to low temperatures *in vivo* while conduction fails at 11°C in other areas of this same nerve maintained normally *in vivo* at much higher temperatures (Chatfield, Lyman and Irving, '53).

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Although the effect of temperature change on nerve function has been the subject of many early investigations on whole frog or mammalian nerve (Lapique, '07; Verzar, '12; Adrian, '14, '21; Schriever, '32) and some recent studies of the single myelinated frog fiber (Tasaki and Fujita, '48, '49, '53; Schoepfli and Erlanger, '41; and Hashimura and Wright, '58) the squid giant axon with the intracellular electrode (Hodgkin and Katz, '49), the reasons for the effects of temperature change remain obscure.

The crustacean nerve fiber has apparently been omitted in temperature effect investigations. It is, of course, to be expected that since the temperature range of the normal habitat of the lobster, Homarus americanus, is similar to that of the squid and certain amphibia, the results of the several investigations mentioned suggest the type of change to be found in the properties of lobster nerve fibers, and in the light of the results of recent detailed studies of these fibers (Wright and Coleman, '54; Wright and Adelman, '54; Wright, Coleman and Adelman, '55), but with no data available from direct observations, the present study was undertaken.

There are several obvious questions which may be answered very simply. These include: What temperature causes conduction block in the crustacean motor axon? Is it possible to initiate oscillatory firing in these fibers at low temperatures? Is the non-oscillatory fiber affected differently than the oscillatory one by cooling? What changes occur in the excitability properties of these fibers at low temperatures? And there are many others.

But the real interest lies in the actual cause of the block itself. One would really like to know what happens in the nerve membrane at low temperatures, what microscopic changes occur to produce the loss in excitability and conduction. The results reported here do suggest certain possibilities which with further experimentation should produce the explanation.

METHODS

The giant motor axons of the walking limbs, the chelae and the ventral nerve cord of the lobster were used throughout these investigations. The isolation of these fibers has been described elsewhere (Wright and Coleman, '54). The isolated nerve fibers were mounted on three electrodes to form a sort of V as described previously (Wright, Coleman and Adelman, '55) and stimulation and recording were accomplished at oil-water interfaces through which the V was raised and lowered. The temperature of the medium was varied by the addition of dry ice or warm water in a moat surrounding the fiber chamber on the microscope stand. It was measured with a very sensitive small mercury bulb thermometer, full scale range 0-45 degrees centigrade, made by the Taylor Instrument Company. The thermometer stem was placed in the fluid immediately adjacent to the cathode or grounded electrode. Since both stimulation and recording took place in this precise area, error due to the temperature gradient between the nerve tissue and thermometer was minimized and the reading considered accurate for the actual nerve fiber temperature to within a fraction of a degree centigrade. Stimulation was effected by Grass S4B stimulators discharging through isolation units, and recordings made without preamplification on a Dumont 322 Twin Beam Oscilloscope. The fibers were isolated and maintained in sea water shipped from the Woods Hole Marine Laboratory or Cole's Physiological Solution (Cole, '41) buffered to Ph 7.6 according to the author's direction. In all, 55 single fibers were used in this study including 14 opener, 16 fast closer, 15 slow closer fibers of walking legs, two fast and two slow closers of the chelae and six giant fibers from the ventral nerve cord.

RESULTS

General. The effects of cooling on the various conduction and excitability properties of a typical highly oscillatory single crustacean fiber, an opener, are illustrated in figure 1. Initially at 24 degrees centigrade the fiber response was oscillatory even at threshold, 85 millivolts, voltage. A frequency of 160 impulses per second was obtained with the stronger 140 millivolt stimulation. As the temperature was lowered this repetitive firing at threshold voltage gradually disappeared becoming completely absent at 15°C, although the oscillatory response was very easily elicitable with the stronger shocks at this temperature. The D.C. threshold (Rheobase) value was markedly decreased by cooling. At 6°C the fiber responded to as little as 48 millivolts D.C. and actually still was capable of firing repetitively at 40 cycles per second. At 4°C the oscillatory nature of the response had completely disappeared and at the same time the threshold value, 52 millivolts, had begun to increase. The threshold value increased to 60 millivolts as the temperature was lowered to 3°C. To offset a rapidly developing block of conduction (note the marked reduction in spike size) and the total loss of the on-off

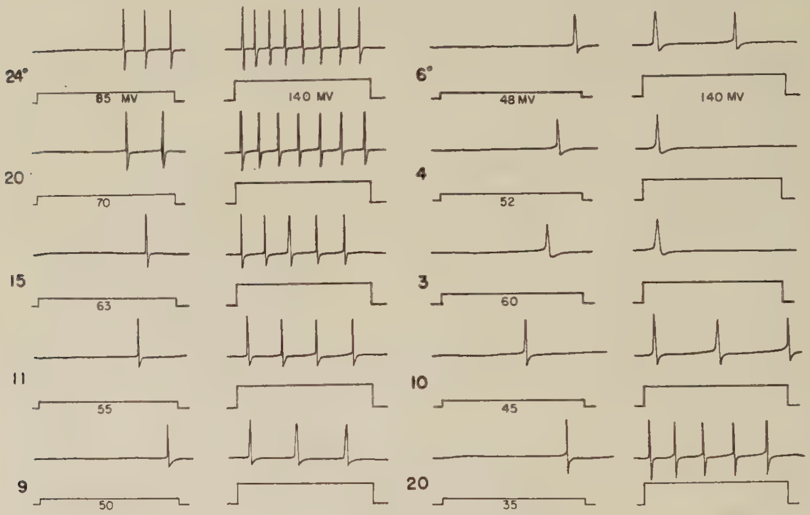

Fig. 1 Action potentials recorded from a typical opener (oscillatory) fiber at different temperatures. Two records, traces immediately to the right of each temperature value, were taken at each temperature, one at threshold (rheobase) stimulus voltage, indicated beneath the trace, the other at 140 millivolts, maximal stimulus voltage, also indicated. The stimulus duration was 50 milliseconds.

effect of stimulation occurring at this temperature or slightly less, rewarming of the fiber was started at this stage of the experiment. The responses produced at 10°C and 20°C during this rewarming process are included in the illustration. Repetitive firing at 60 cycles per second was elicitable at 10°C and 100 cycles per second at 20°C by the 140 millivolt shock. It is interesting to note that the final threshold value, only 35 millivolts at 20°C had remained well below the initial value. This difference between pre and post cooling threshold values was almost always seen. The return to the original value was often extremely slow, sometimes requiring several hours.

Throughout the early period of temperature reduction the utilization time as indicated by the latency of firing at threshold remained constant or was slightly increased. But at very low temperatures, coincident with the threshold rise the utilization time decreased markedly. This change was still evident during the early stages of rewarming, 10°C, but at 20°C the normal long latency time for this fiber type was again recorded. On the other hand, there is a several fold increase in the minimum latency time during cooling, as measured by the distance between the rising edge of the 140 millivolt stimulus artifact and the foot of the first or only spike of the volley elicited. This means that the velocity of conduction was decreased by a factor, in this case, of about three.

The action potential wave shape changed considerably during cooling. This can be more clearly seen in figure 2, a highly amplified recording of just one spike of the volleys in figure 1. As the temperature was reduced to 11°C the voltage amplitude changed very little, but from 11°C to 3°C the spike height became less than half the original amplitude. The duration was lengthened at 3°C to almost three times the original value. With rewarming of the fiber, the action potential rapidly regained its original size and shape.

From this general survey of the effect of cooling on the excitation and electrical properties of the single crustacean nerve fiber, several conclusions may be drawn immediately. First, repetitive firing can be elicited at low temperatures but at frequencies much lower than normal. Second, the rheobase value is somewhat reduced by cooling as has been shown in nerves of other species (Gasser, '31). Third, the utilization time remains unchanged or is slightly increased except at very low temperatures; and fourth, the velocity of conduction is

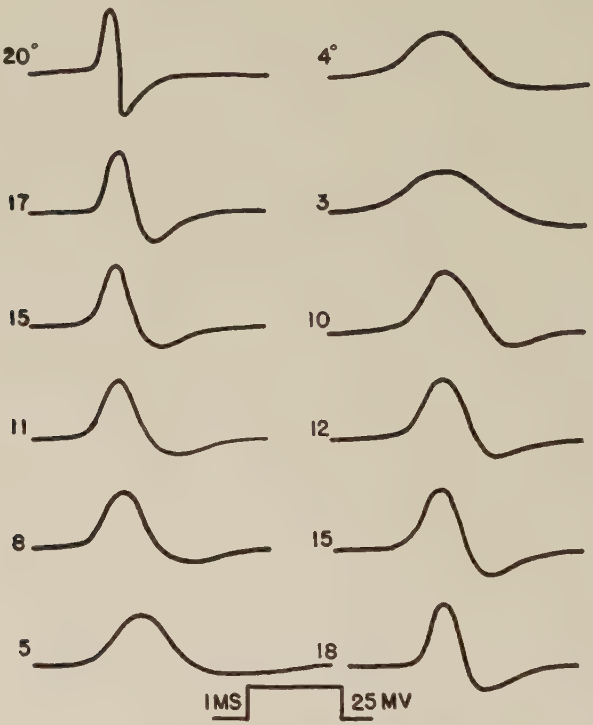

Fig. 2 Action potential recording at fast sweep speed from the same opener fiber as in figure 1 showing only one spike of the volley, initiated at each temperature listed at the left of each trace. Twenty-five millivolt one millisecond duration calibration trace.

decreased. The first three conclusions suggest accomodation has been reduced at moderately low temperatures in accord with earlier findings (Schriever, '32; Tasaki, '49; Solandt, '36), because a low accommodation level is to be associated with low rheobase, long utilization time and of course oscillatory firing (Wright, Coleman and Adelman, '55). The reduction of oscillatory frequency indicates increased refractory period or recovery time as found in frog nerve at low temperatures (Gasser, '31; Adrian, '21). Indeed, if it is born in mind that the second spike of a volley, is always slightly smaller than the first (Hodgkin, '38) it is interesting to note that this relative difference in spike size is the same no matter what the temperature, note the recording at 6°, even though the time interval between the two spikes has greatly increased. The second spike must still be initiated in the same refractory phase of excitability, only the refractory period has become significantly longer.

A fifth conclusion is that at exceedingly low temperature, near freezing (2–3°), where the rheobase value increases, the oscillatory response disappears and the utilization time becomes short, accommodation must increase markedly to account for these changes. Thus cooling may at first cause a decrease in accommodation, but later with extreme cold accommodation becomes a significant factor. In order to substantiate this and the other conclusions, many accurate measurements of rheobase values, excitability rate constant, k, values and accommodation time constant, λ, values have been obtained at different temperatures.

Excitability. The strength duration curves of the slow closer fiber at 23°, 17°, 6° and again at 20°C are shown in figure 3. These curves and all the following excitability data recorded below were obtained directly at the cathode with the fiber raised in oil. During cooling the rheobase value reduced, in this case from 150 mv. to 100 mv., but at the same time the threshold for short duration shocks was increased. This is shown too by the increased chronaxie values, arrows, at low temperatures. The chronaxie, approximately 2 ms at 20°C lengthened to 3 ms at 6°C. Thus cooling caused the strength-duration curve to be lowered and moved to the right relative to the coordinate axes, or, in other words, the tissue became more easily excited with longer duration impulses, less easily excited with short duration pulses. This immediately suggests that two fundamental changes are taking place. First, the local excitatory state must rise more slowly in the cold; i.e., Blair's rate constant (Blair, '32), k, must be reduced, and therefore the excitatory process may not reach threshold level during the period of a short duration shock. Second, the threshold itself must be prevented from increasing, even slowly; i.e., accommodation has decreased, and hence appears to be slightly lowered. The rate constant, Blair's k, values are included in the figure as obtained directly from the chronaxie value or obtained from Blair's equation, k_{calc}, and indeed, these values do decrease on the average 30–40% with a twenty degree temperature reduction from 25°–5°C. Furthermore, the strength-duration curves of a typical opener fiber at 24°C and 6.5°C, shown in figure 4, exhibit the sort of "cross-over"

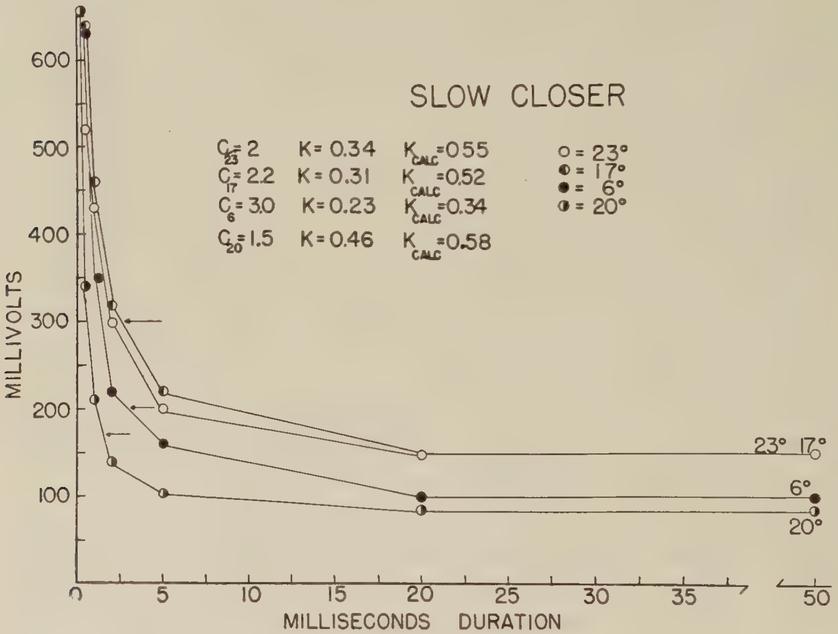

Fig. 3 Strength-duration curves of a slow close fiber at 23°, 17°, 6° and 20°C as indicated in the figure. Chronaxie values (C), excitability rate constant values taken from chronaxie values, K = log 2/C, and rate constant values calculated K_{calc}, from the curve data, Kt = log (V/V-R), (15), are listed. See text.

already reported for fibers with different accomodation time constant values. Nerve fibers with little or no accommodation usually have a strength-duration curve with the lower rheobase value shaped as the 6.5°C curve in the figure whereas the curves of fibers with considerable accommodation have higher rheobase values and a more acute curvature as in the 24°C data shown in the figure.

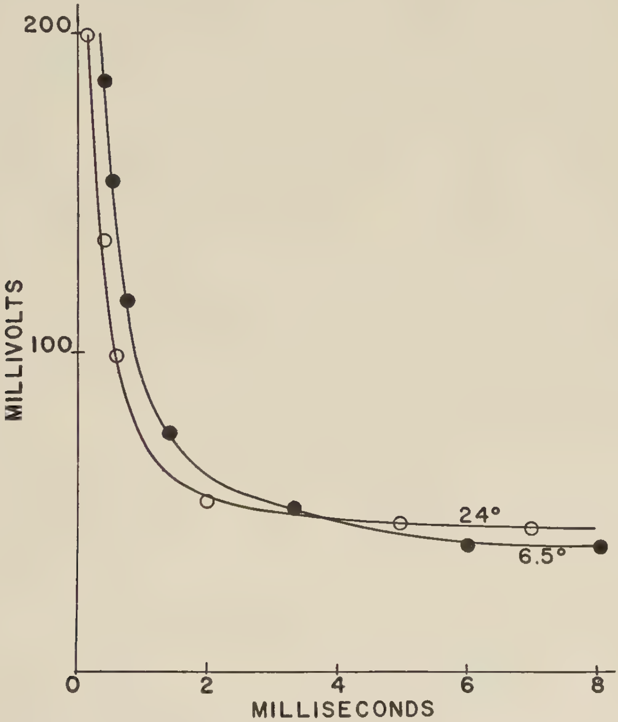

Fig. 4 Strength-duration curves of an opener fiber at 24°C (open circles) and at 6.5°C (solid circles).

Theoretically, accommodation can be evaluated by using an exponentially increasing stimulating current (Wright and Adelman, '54; Solandt, '36; Hill, '36); but this method has certain limitations, one of which becomes a nuisance at low temperatures. If measured in this way, figure 5, the time constant of accommodation decreased in value with cooling especially at very low temperatures, a totally unexpected result. The mean value of λ obtained by this method from 11 oscillatory fibers decreased significantly up to 90% for all lower than normal temperatures, but the value from seven non-oscillatory fibers increased up to 15% for temperatures down to 12°, but declined markedly with further cooling. In order for this method to work, the time constant of the local excitatory state, the inverse of k, must remain small (Hill, '36), but at low temperatures it is greatly enlarged. Therefore, only those results obtained at higher than 15° may be considered

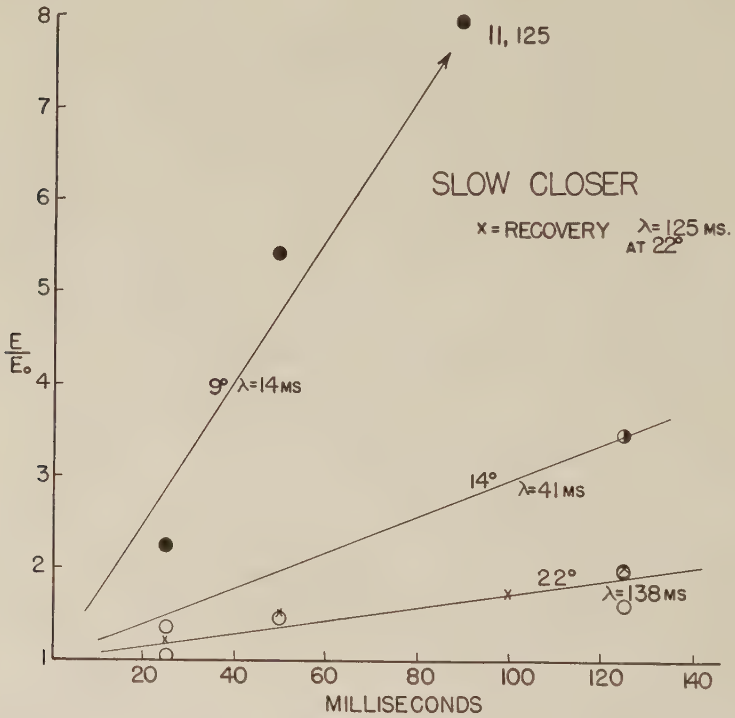

Fig. 5 Accommodation measurements of a slow closer fiber at 22°C (open circles), 14°C (half filled circles), 9°C (solid circles) and 22°C (X's). The time-constant in milliseconds of an applied exponential current is plotted (abscissa) against the ratio of E, the voltage required to excite at the time constant chosen, to E_o, the rheobase voltage. Plotted in this way, the value of the accommodation time constant, λ, is given by the slope of the straight line.

valid, and some of these did show a slight decrease in accommodation as anticipated.

The actual process of accommodation can best be illustrated by the dual shock technique (Wright, Coleman and Adelman, '55; Le Fevre, '50) although no exact value can be obtained by this method. Results with this technique are shown in figures 6a, b, c, d. An opener fiber which responded non-repetitively, NR, (curve marked x's, fig. 6a) at 21°C was cooled to 14°C (curve #1), then to 5°C and then rewarmed to 14°C (curve #2) and finally 21°C. There was a marked decrease in threshold at low temperatures during the first 4 ms followed by a much more rapid rise than seen at 21°C. The establishment of "perfect" accommodation (Wright, Coleman and Adelman, '55; Le Fevre, '50) or 100% normal value occurred after 15 ms. The rewarmed fiber responded repetitively, R, at 21°C and the threshold value remained fairly constant with very little accommodation, at the new low value attained during the first few ms at 5°C. There is no question in this case that accommodation increased in the cold. This was even more clearly seen when the experiment was repeated on the same preparation about one hour later, figure 6b at 23°C, curve #1, the fiber response was highly repetitive, R, and the threshold value remained steady after the initial drop, indicating very little if any accommodation. But at 2.5°C the threshold rise after 7 ms was rapid to the perfect accommodation level. The excitability curves became even more acute after holding the fiber at this temperature for ten minutes. The initial threshold value declined actually to the zero or spontaneous firing level during the conditioning pulse but then extremely rapid accommodation occurred after only 5 ms and prevented the appearance of spontaneous discharges. Lengthened refractory period (see below) also inhibited repetitive firing in this brief interval.

Excitability changes were more often as shown in figures 6c and d. The threshold of excitation of the opener fiber, figure 6c, which responded repetitively, R, at 23°C (X's) was markedly reduced almost 30 ms at 5°C, at which temperature

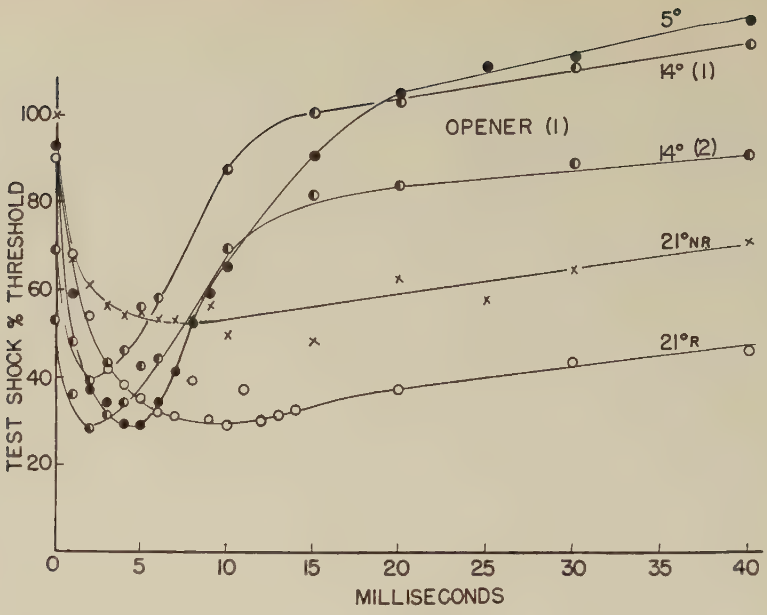

Fig. 6a Excitability curves of opener fiber obtained by the dual shock method at 21°C (NR means non-repetitive at this time), 14°C (1 indicates first measurement at 14°C), 5°C, 14°C again (2 means second measurement at 14°C during rewarming) and 21°C (R indicates fiber is now capable of repetitive firing). See text.

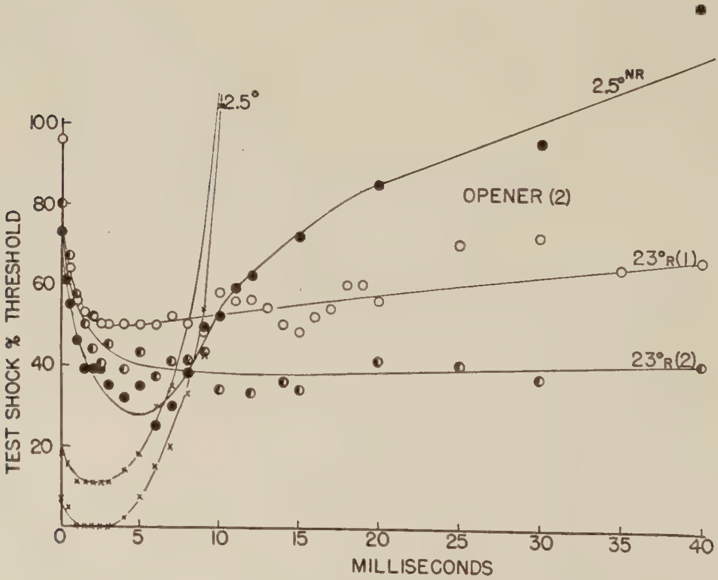

Fig. 6b Excitability curves from the same fiber recorded one hour later. See text.

the fiber became non-repetitive (NR) in response. Slow accommodation occurred during the next 35 ms. Even at 2°C the threshold remained remarkably low for 15 ms after which a pronounced increase in threshold value indicated fairly

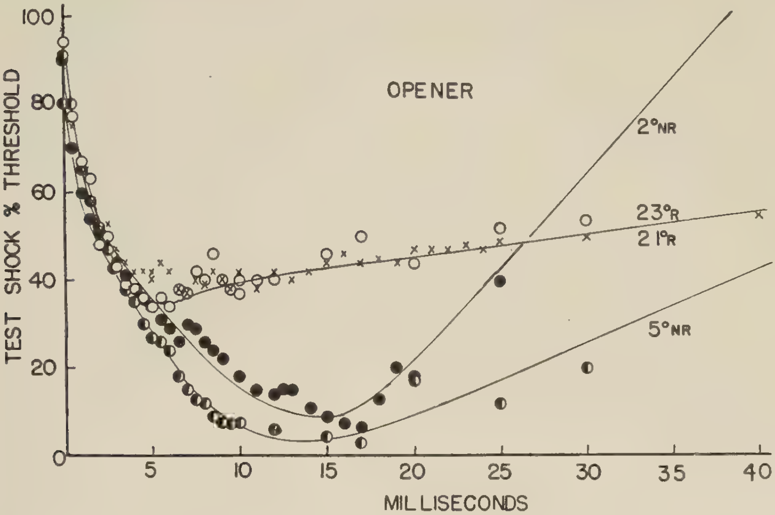

Fig. 6c Excitability curves of opener fiber at 23°C, 5°C, 2°C and 21°C. See text.

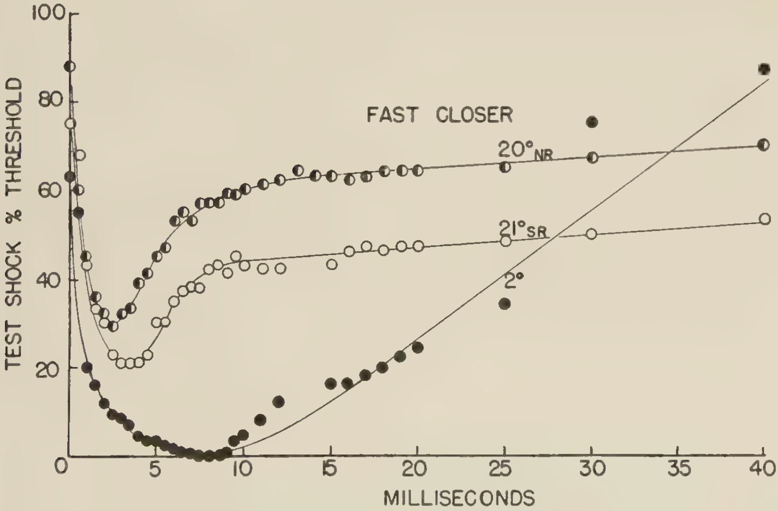

Fig. 6d Excitability curves of fast closer fiber at 21°C, 2°C and 20°C. See text.

rapid accommodation was taking place. With rewarming to 21°C (circles) the fiber regained the original excitability properties. When cooled to 2°C the subliminal voltage caused the threshold of a fast closer, figure 6d, to fall zero for 10 ms and then to rise almost linearly to the perfect accommodation level after 40 ms which was a higher threshold value than recorded either before or after cooling. Upon rewarming, the fiber was no longer capable of firing repetitively, NR, because it had become somewhat less excitable during the course of the experiment. This is indicated by the position of the final excitability curve above the original one. From these data it must be concluded that accommodation is increased by cold, especially intense cold, although the rate of accommodating and the onset time may be considerably slowed.

Another interesting fact worth pointing out here is that the values of the threshold actually fluctuate about the mean curve, figures 6a and b, indicating an oscillation of threshold at a frequency of about 100/sec. at the start of the experiment, X's, and 200/sec., O's, at the end of the experiment. This oscillation is clearly seen in figure 6b. In this case the threshold values were obtained at more frequent intervals from the same fiber. Thus the oscillatory nature of the fiber, whether capable of responding repetitively, figure 6b, 6c, or not figure 6a, may be observed in the threshold value fluctuations, and it will be noted that these, too, are suppressed at low temperatures.

The recovery of excitability following a discharge differs between fibers (Wright, '56) and is actually another means of distinguishing fiber types (Hodgkin, '48). Normally, the highly repetitive firing opener fiber enters into a transient hyperexcitable state of about 10 ms duration, figure 7b (open circles), following the refractory period. This super-normal phase results in a dip in the threshold value curve (open circles) below the final threshold value indicated by the straight reference line at 21°C.

This super-normal phase is much less pronounced if it occurs at all during the recovery phase of the larger, less oscillatory, fibers such as the fast closer, figure 7a. At low tem-

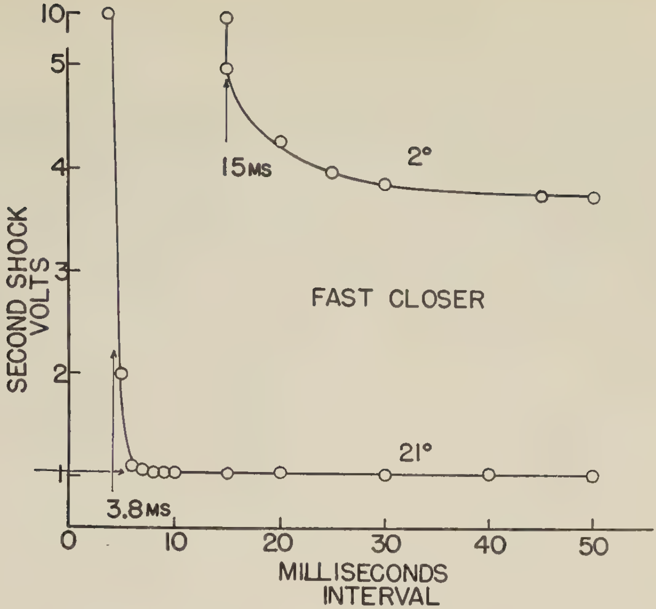

Fig. 7a Excitability recovery curves of a fast closer fiber at 21°C and at 2°C. Two short duration (0.2 ms) stimuli were applied separated by the interval indicated by the abscissa. The threshold value of the second shock is indicated by the ordinate. See text.

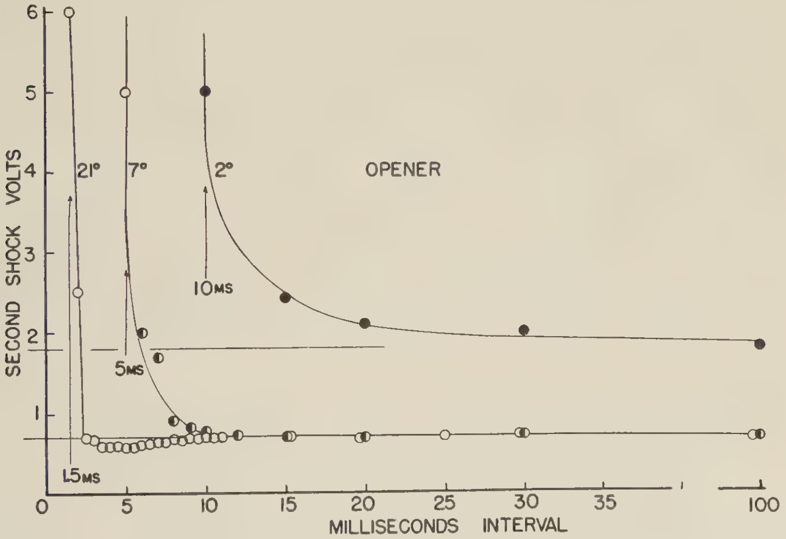

Fig. 7b Excitability recovery curves of an opener fiber at 21°C (open circles), 7° (half filled circles) and 2°C (solid circles). Note the super-normal state at 21°C as shown by dip of open circles below the straight reference line drawn through the final threshold value. This dip is absent at lower temperatures.

perature the curves were displaced upwards and to the right relative to the coordinate axes. The upward shift was due to the rise in threshold value for the very short duration, 0.2 ms, stimulus used to examine the recovery process. The displacement to the right was due to a very sizeable increase in the refractory period. The absolute period of the fast closer was 3.8 ms at 21°C, 5 ms at 7°C and 10 ms at 2°C. Absolute refractory periods of 30 ms have been observed and relative refractory states may last several hundred milliseconds at low temperatures. Similar large increases in the refractory period time during cooling has been recorded in frog nerve (Gasser, '31). Typical recordings from an experiment designed to measure the recovery of excitability are shown in figure 8.

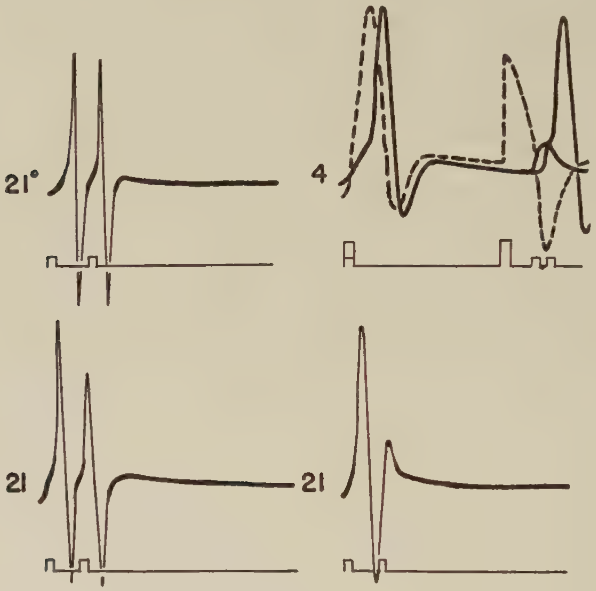

Fig. 8 Action potentials recorded from an opener at 21°C and at 4°C, elicited by two short duration (0.4 ms) pulses separated by the least interval for above threshold voltage response upper and lower left at 21°C. The upper right picture shows the least interval for above threshold voltage response at 4°C (solid line trace), for supra-maximal shocks (dashed line trace). The lower right picture shows least interval for response extinction at above threshold voltage.

The action potentials elicited by two 0.4 ms duration shocks (traced below each recording) and recorded from a single opener fiber at 21°C and at 4°C. The separation between the shocks shows the absolute refractory period or the least interval for this fiber at these temperatures. At 21°C, at the start of the experiment, upper left picture, the least interval for just above threshold stimulus was 2 ms. At 4°C, upper right picture, it was 8 ms, a fourfold increase. Only the local response was recorded with a slightly smaller interval as shown in the figure. With four times threshold stimulation, dashed lines, the least interval was reduced to 7 ms, but much of the second action potential was obscured by the large shock artifact making accurate measurement impossible. When rewarming to 21°C again, lower pictures, the least interval was 1.5 ms, left, or 1.3 ms, right, with the second spike just recordable or just extinguished by the absolute refractory state of the first, respectively.

It has been established that the value of the theoretical excitability constant, k, is decreased by cooling. This means that the rate of decay of the local excitatory state is reduced by cold, if the theory is correct. If the first of the two short duration shocks is just sub threshold strength so as to initiate only the local response, a study of the decay of the local excitatory state can be made by recording the threshold values of a second shock at various intervals after the first. The data obtained from an opener fiber at 23°C and 6°C are shown in figure 9. The absolute voltage or amplitude values were very different, and hence were made comparable by the use of the percent excitability scale on the ordinate. It is clear that the normal decay of excitability (open circles) following a sub threshold short duration shock was markedly slowed by cooling (solid dots) as shown by the much more gradual slope of the latter curve. Thus at time zero, as the conditioning shock was just turned off, the excitatory state was 95% of threshold value and a second shock strength only 5% threshold value for that particular temperature was required to summate and excite. At 23°C the excitatory state fell in one millisecond from this 95% level to only 6%, requiring a second shock of 94% threshold strength to summate. In the same time interval at 6°C the excitatory state declined about 60% to only 35% threshold value so that a much weaker second stimulus of only 65% threshold strength was able to excite. Actually the two curves drawn in the graph are from the simple exponential equation $E = E_0e^{-at}$ and the fit of these theoretical curves to the data is good, using the two different values for "a," 2.5 at 23°C and 1.1 at 6°C as shown on the figure. Thus Blair's k value is represented by "a" in this case, and although it is rather unusual to find a k value as high as 2.5 in this type of fiber, opener, this value is of the right order of magnitude. Certainly the 55% decrease in this value with cooling is not out of line with the 30-40% decline in the k value already reported above.

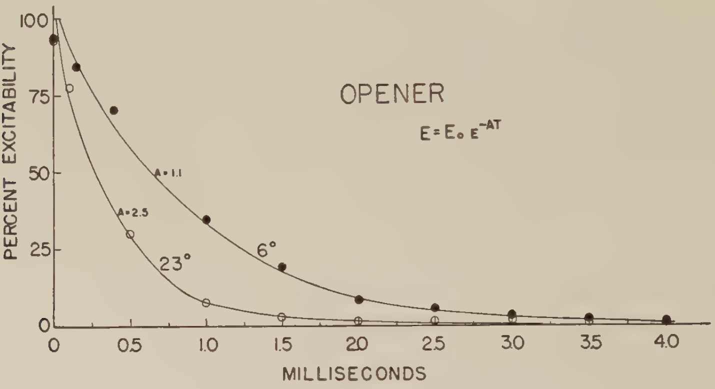

Fig. 9 The excitatory state of an opener fiber following a just sub-threshold short duration stimulus at 23°C (open circles) and at 6°C (solid circles). The values of the second shock, applied at intervals after the first shown in the abscissa, are plotted, ordinate, in percent of the threshold value for this stimulus applied alone. The curves are from the equation $E = E_0e^{-at}$.

Electrical. Before discussing the significance and possible meaning of these findings with regard to the changes occurring in the functional mechanism of the cooled fiber, it is important to note the effects of low temperatures on the electrical properties of the fiber. For while crustacean single nerve fibers undergo the changes in excitability described above when cooled, the electrical properties of the fibers are also affected by lowering the temperature. The change in the conducted diphasic action potential wave shape has already been shown, figure 2. The monophasic spike of a typical slow closer fiber conducting over a distance of about 10 millimeters at different temperatures is shown in figure 10. The duration of the stimulus was 0.2 ms and the strength was set just above threshold value at each temperature. The velocity calculated from the delay time was initially 9 M/sec at 21°C and reduced to 2.5 M/sec at 6°C, although a stronger stimulus at this temperature resulted in an increase in the velocity of conduction by a factor of 2 as indicated by the deflection with the shorter delay time.

This apparent increase in velocity is probably not due to excitation being initiated in a different region of the fiber nearer the recording lead. It is most likely the result of a marked latency decrease at the point of excitation. At 5°C a very broad all or none response was elicitable, but at temperatures below this value the all or none response disappeared leaving only an extremely long enduring non-conducted low voltage, the local potential. It is normally impossible to record this response at such a great distance from the point of stimulation. Evidently as the temperature approached the blocking value the local potential was so greatly enlarged in voltage, duration and spatial spread (see below) that it was detectable at some distance from its origin. With rewarming the all-or-none response reappeared at 8°C at which temperature the local response was no longer recordable. The original spike voltage and duration were obtained as the temperature approached 20°C. In general the larger non-oscillatory fibers became non-conducting at 4–6°C whereas the smaller oscillatory fibers blocked at 1–3°C.

If the spike deflection is assumed to approxmiate a simple triangle and the area under the trace of the spikes measured at the different temperatures, it is found that the area of the spike at 6°C, figure 10, was almost double that at 21°C. In absolute units the spike at 21°C was 45 mv and 0.9 ms duration at the base or the area under the trace was 200 millivolt-milliseconds. At 6°C the spike height was only 25 mv while the duration was 40 ms, so that an area of 500 millivolt-milliseconds is contained. Taking into account the large local potential which contributes about 200 mv-ms to the total, the actual change in the spike is about a 50% increase in total "quantity" of electricity. The actual spike voltage was never observed to enlarge significantly during cooling with the present method of recording with external leads. This has been found also for frog nerve fiber spikes (Tasaki and Fujita, '48), al-

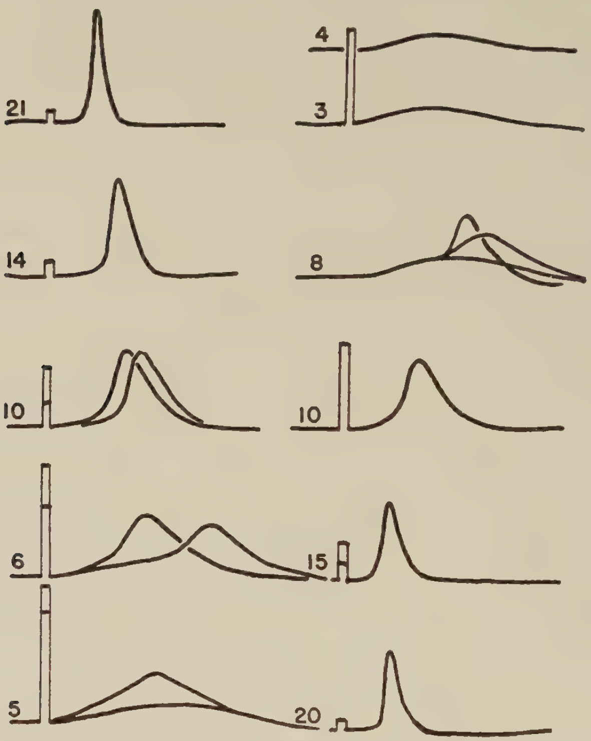

Fig. 10 Monophasic action potentials recorded at the temperatures listed from a slow closer partly in oil. The conduction distance between oil-water interfaces was 10 millimeters. The 0.2 millisecond duration shock is shown. The relative amplitude of the shock in the case two different voltages were applied is given by the line across the trace for the weaker of the two.

though the exact opposite has also been recorded with cooling of frog fibers (Schoepfli and Erlanger, '41). With the intracellular electrode a small increase in the action potential has been recorded from the squid giant axon during cooling (Hodgkin and Katz, '49).

The appearance of the large non-conducted potential at low temperatures was investigated directly by recording from the same fiber at the cathode at various temperatures, figure 11. The three recordings at each temperature (left to right) were

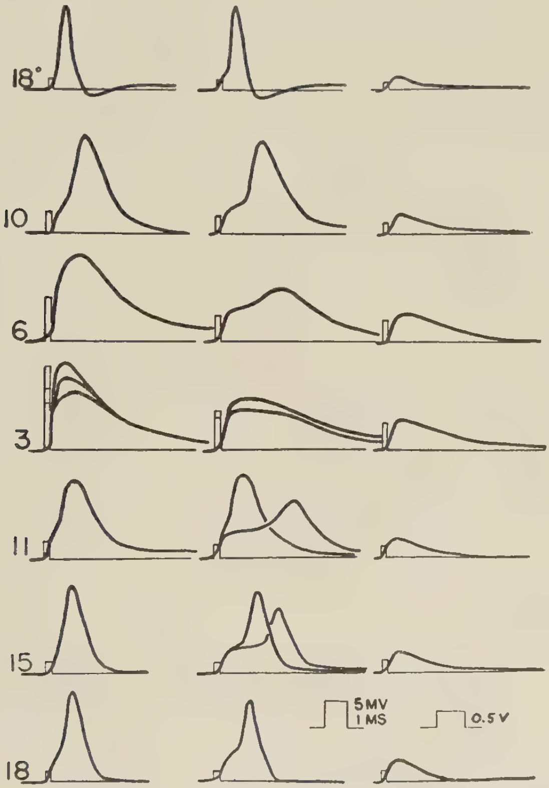

Fig. 11 Action potentials recorded at the temperatures listed from the same slow closer (fig. 12) raised in oil. Three recordings made at each temperature were elicited by maximal, threshold and just sub-threshold strength 0.2 ms duration stimuli, left to right. 5 millivolt, 1 millisecond calibration for the action potentials and 0.5 volt calibration for stimulus trace.

initiated by maximal, threshold and just subthreshold short duration stimuli. As the temperature was lowered the subthreshold response became very much larger both in voltage and duration, right column, as the threshold value to short duration stimuli increased and a higher voltage shock was applied. The spike became tremendously broadened at 10°C and the latency increase with threshold stimulation, middle column, was especially noticable. Beginning at 6° a peculiar change occurred. The all or none response of the fiber ceased. Although a small all or none response ''popped'' on and off and was distinguishable with threshold stimulus at 6°, it became completely obscured by the tremendous increase in amplitude of the local response with above threshold stimulation voltage. At 3° it had disappeared completely. The response now generated by the applied voltage varied in magnitude with the stimulus strength, but apparently within certain rather wide limits. Certainly the local response could become so large with strong stimulus voltage as to spread down the fiber and be recorded at some distance from its origin, figure 10. When the fiber was rewarmed this great enhancement of the local response disappeared, and the spike reappeared. The large latency of the spike at low temperature must account in part at least for the markedly increased ''conduction time'' with low voltage stimulation and hence must be taken into consideration in the calculation of the velocity, as already pointed out. It will be noted that the recording at 18°C finally had become very nearly as it was at the start of the experiment. This reversibility of the cold effect was only possible if the fibers were not subjected to very low temperatures for long periods of time, only a few minutes. This was found to be especially true for the non-oscillatory fibers. Otherwise, upon rewarming a partial recovery would be followed by a rapid and progressive loss of excitability.

The loss of the on-off mechanism of the spike at very low temperatures below 3°C, as recorded from a typical fast closer fiber, is illustrated in figure 12. Fibers of this type usually became non-conductive at slightly higher temperatures and therefore the effect was easier to obtain. All the tracings were recorded directly at the cathode except the upper left picture which was made with the recording lead 10 mms from the cathode to show the enormous spread of the local potential. These potentials are readily distinguishable from shock artefacts primarily because of the rounded off peak and the relatively slow decay compared with the shock deflection.

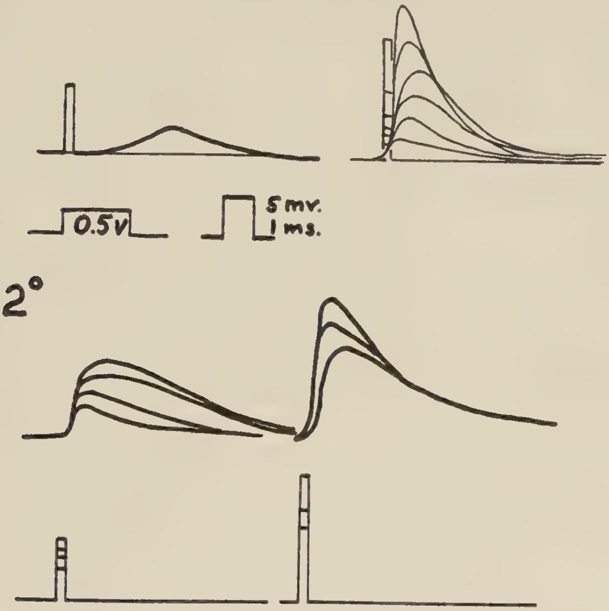

Fig. 12 The response of a fast closer fiber at 2°C when stimulated by a 0.2 millisecond shock of relative strength indicated by the lines across the shock trace. The fiber was partly immersed in physiological solution, upper left, so recording was made about 10 millimeters from point of stimulation. All other traces obtained from fiber raised in oil.

To examine the effect of cold on the local response in more detail, the local responses of the opener fiber, figure 11, elicited at 18°C and 3°C have been magnified and traced in figure 13. To make the curves comparable, the percent amplitude scale was used on the ordinate, the regular time scale in milliseconds on the abscissa. The 18°C curve rises more rapidly reaching

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100% amplitude in .4 ms as compared to .5 ms at the 3°C temperature. However, it will be noted that the slopes of the rising phase do not begin to differ until the potentials have attained about 60% amplitude, and since the potential at 3°C is almost double the voltage of the potential developed at 18°C (see fig. 11), the slope difference occurs during the rounding off of the rising phase at voltages well above that of the peak voltage of the higher temperature curve. At 18°C the local response decay is exponential and follows the equation $E = E_0 e^{-at}$ with $a = 1.3$ (triangles) which is a reasonable value for the rate constant of the opener fiber. However, neither $a = 0.6$ (open circles) nor $a = 0.8$ (X's) when substituted in the equation give a good fit to the decay curve at 3°C. In fact, part of this decay curve appears to be nearly linear. A

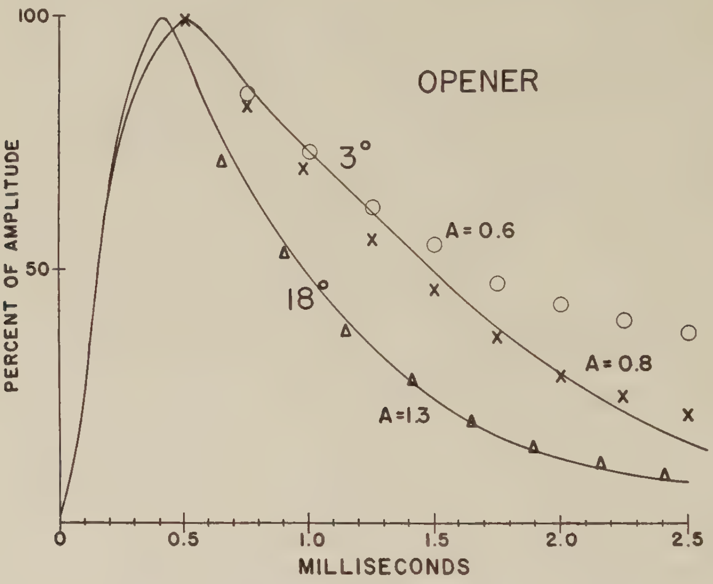

Fig. 13 Sub-threshold response (lines) of opener fiber at 18°C and 3°C. The amplitude (ordinate) is in percent rather than volts to make the two curves of comparable size so that the respective decays may be examined. The values of the Δ 's were obtained from $E = E_0 e^{-at}$ with $a = 1.3$; for open circles $a = 0.6$ and for X's, $a = 0.8$.

better illustration of this is shown in figure 14 in which the local responses of the fast closer (fig. 12) have been similarly amplified. One of the curves is the tracing of the local response at 18°C while the other three were traced from recordings made at 2°C with three different stimulus voltages of increasing amplitude. The rising phase begins earlier and is steeper at 18°C than at 2°, the voltage peak being attained in 0.6 ms as compared to 0.8, 0.9 and 1.1 ms with weak, medium and strong stimuli for the cold fiber. Again the reason that the response to the strongest stimulus (uppermost curve) requires the longest time to reach the peak voltage is because of the rounding off of the rising phase and indeed, there is the barest indication of a slight all or none after 1.5 ms. The rapid decay of the local response at 18°C is well fitted by the exponential equation with the value 0.95 = a (solid circles). However, the falling phase of the potentials at 2°C does not conform as well with the exponential equation for any value of a, the closest fit being obtained with a = 0.4. A large portion of the curve is linear and the linear portion of all three potential curves are very nearly parallel. The straight line given by E = –bt were b = 6 (triangle) and b = 5.5 (X's) fits the decay phase quite well. However, the tails of the potentials from 3.5 ms

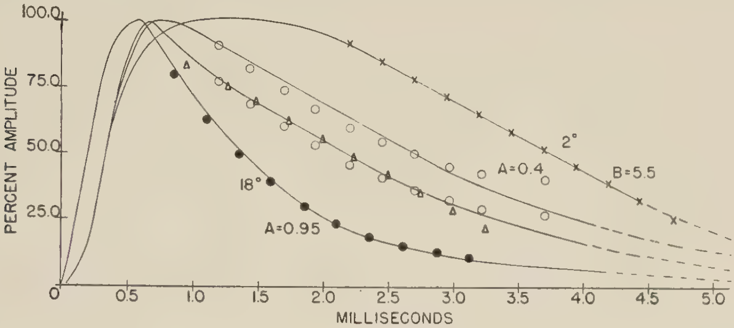

Fig. 14 Sub-threshold response of fast closer fiber at 18°C and elicited by weak, medium and strong stimuli at 2°C. The exponential $E = E_0e^{-at}$ values for a = 0.95 (solid circles) fit the 18°C curve, but for a = 0.4 do not fit the 2°C curves. The straight-line equation E = –Bt, where B = 6 (△'s) and 5.5 (X's) fits part of slopes at 2°C.

on out do curve exponentially and can be reasonably well fitted by substituting the value $a = 0.7$.

To study in detail the conduction rate variations of the propagated response as well as the wave shape, the action potentials of the opener fiber (fig. 11) at 20°C and 6°C have been enlarged and traced, figure 15, solid lines. The spikes were elicited by 0.2 ms stimuli of slightly above threshold strength. With stronger stimulus strength, dashed lines, the latencies are markedly reduced. This suggests either a greater velocity of conduction with the stronger shock, stimulation at a point further along the fiber, or reduction of delay at the cathode. Recording directly at the cathode shows that the last possibility is the most likely cause of the apparent conduction rate change. The latency at the cathode at 20° is 0.4 ms, La, whereas at 6°, L_b, it is almost 2 ms. With very strong stimulus these latencies may be reduced to 0.2–0.4 ms which give a corrected value of 13 M/sec instead of 9.5 M/sec at 20°C, and 4 M/sec instead of 2.5 M/sec at 6°. This certainly minimizes but does not rule out the effect of some electronic spread along the fiber with strong stimuli.

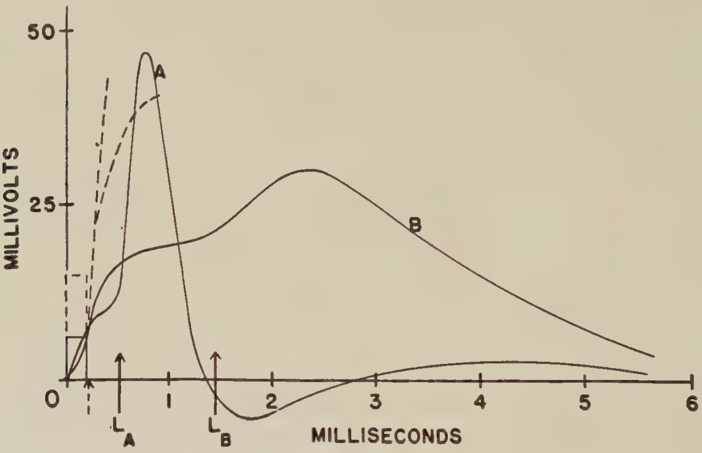

Fig. 15 Action potentials of opener fibre of figure 11, recorded with fiber in oil. A is spike at 20°C, B at 6°C for threshold stimulation, dashed lines show rising phases of A and B with strong stimulation. See text.

At normal temperature the delay is shortened to a minimum by stimulus strength increase of only 10-20% above threshold value whereas it requires a much larger increase, up to 100% or more above threshold, to accomplish the same latency decrease in the cold fiber.

Another factor to be considered is the wave shape. It is impossible to show the exact wave shape of the all or none response without applying a longitudinal current clamp and recording the fiber response with zero net membrane current (Marmont, '49). However, if the rate of change of the local response is reduced it is not unreasonable to assume that the rate of rise and fall of the spike phase is similarly retarded by cooling. A simple arithmetical analysis can be carried out which shows this to be the case. If one pictures the approaching spike potential as "seen" by an external electrode as consisting of a sequence of brief square waves, small to large, separated by brief time intervals arbitrarily chosen, the rising phase of the spike can be approximated (open circles) using the normal velocity value of 13 M/sec, figure 16. But to fit the

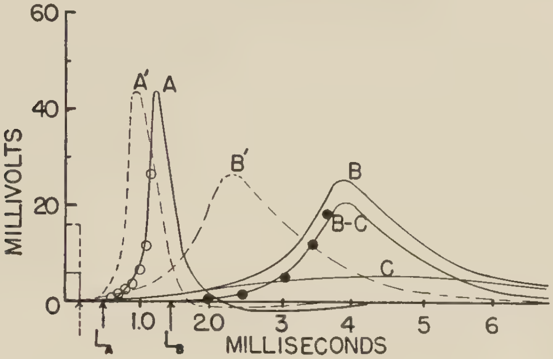

Fig. 16 Action potentials of opener fiber at 20°C and 6°C recorded with fiber partially immersed. Conduction distance about 10 millimeters. Solid lines are potentials elicited by just above threshold stimulus, 50 millivolts, 0.2 millisecond duration. Dashed lines (A′, B′) spikes elicited by 180 millivolts. C is local response recorded at distance of 10 millimeters with sub-threshold stimulus. B-C is spike along at 6°C with local response subtracted. See text (discussion) for explanation of plotted circles and dots.

tracing of the spike at low temperature, curve B-C solid circles, it is necessary to not only use the reduced velocity value, 4 M/sec, but also increase the time intervals separating the square waves by a factor of 5. Since the envelope of these waves represents the action potential this means that this curve has changed shape in the sense that it has broadened, or the slope of the rising phase has decreased. This would also tend to increase the time constant of excitation, the utilization time, and reduce the velocity of conduction even more.

Considerations. All of the data presented may be accounted for if one assumes three basic changes occurring in the nerve membrane when cooled. These changes are (1) That the resistance, electrical, of the medium or membrane is increased; (2) the extrusion mechanism for sodium is slowed or blocked entirely; and (3) the membrane viscosity increases.

The strength-duration data tell us that the k value is decreased 40% or by a factor of 1.6 when the temperature is lowered from 25° to 5°C. A saturated NaCl solution has a conductance value of 0.2513 at 25°C, 0.155 at 5°C, or in other words is decreased over the same temperature range by a factor of 1.65. This then may account for the change in the excitability constant and the utilization time, which increases with cooling.

Obviously, if the utilization time is increased the velocity of conduction will be slowed, and one might expect by an equal amount, yet the above measurements show the velocity reduced, for example, from 13 M/sec to 4 M/sec after correction for the delay at the cathode. This is a decrease by a factor of 3.3 between these same temperature extremes. Since the spike is a limited voltage and hence current source, any increase in medium resistance should reduce the amplitude of the exciting currents preceding the spike. Strength-duration data obtained at about these temperature values, 24° and 6°C (fig. 4), give a utilization time at the 100-150 mv level, or spike amplitude, of about 0.3 and 0.6 ms respectively or an increase by a factor of two. If at the same time the spike currents are reduced by a factor of 1.6, the time required to stimulate at any point along the fiber could be increased by as much as 3.2 times taking into account both utilization time and current magnitude changes. The velocity would of course be reduced accordingly.

The slight rheobase reduction, utilization time increase and oscillatory firing at low temperatures suggest an accommodation decrease (Wright and Coleman, '54). The threshold is slower to "move" to a new level during excitation. This may be the result of a slightly increased viscosity in the early and milder stages of cooling retarding the reorientation of membrane molecules or even causing the membrane to become less "leaky" than normal. It has been concluded (Wright, Coleman and Adelman, '55) that accommodation is to be associated with the porous or leaky membrane. Indeed, the accommodation time constant, λ, when measured in nerve tissue with normally low values for λ, (Schriever, '32; Tasaki, '49; Solandt, '36) such as the non-oscillatory fast closer fiber, has been found to increase in value slightly with cooling. The λ value decreased markedly at low temperatures, however, in all fibers with normally little or no accommodation. This very large change in the accommodation time constant is because the method used becomes inaccurate for the following reasons:

The exponential current form is given by $I = I_0(1 - e^{-at})$ where a, the rate constant, $= 1/RC$ where RC is the time constant. If such a current is applied to an electrical system equivalent to a nerve fiber (25) containing a resistance and capacitance the final current form will be $I_1 = I(1 - e^{-kt})$ or $I_1 = I_0 (1 - e^{-kt} - e^{-at} + e^{-t(a-k)})$ where k is the nerve rate constant. If k is large, e^{-kt} becomes negligible and the current through the fiber is the same as the applied current and a measure of the fiber time constant can be obtained from the value of the "a" of the applied current. If k is small as it is, comparatively, in the cold fiber then e^{-kt} becomes an important factor and the current in the fiber is very different from the applied current. It may rise very slowly and in order to reach threshold, assuming some accommodation, a much stronger applied current may have to be used to stimulate, leading one to believe that the value has been reduced.

The dual shock method which is the study of the effect of a sub-threshold D. C. shock on the threshold of a short duration pulse is a good way of avoiding the exponential current difficulties. This method shows that accommodation, as indicated by a rise in threshold during the applied DC, very definitely does increase during cooling, but both the onset and rate are retarded. Thus, the threshold may decrease at first to a much lower value than normal, but then will increase often to the perfect accommodation level or greater. This suggests that under normal conditions some event which takes place quickly after the start of the DC pulse tends to counteract and just balance out the threshold decrease in the oscillatory fiber or cause it to rapidly increase in the non-oscillatory type. At low temperatures this ''reaction'' is delayed allowing the threshold drop to get a ''headstart,'' but after a relatively long latency accommodation does occur. The suggestion that accommodation is due to ionic leaks (Wright, Coleman and Adelman, '55) leads one to wonder if, with cooling this ''turn-off mechanism,'' accommodation, is not only delayed but allowed to build up continuously with time because of slowing of the sodium pump. This would certainly allow the accumulation of sodium within the fiber and displace potassium out of the fiber.

At extremely cold temperatures the action potential spike becomes smaller in voltage amplitude and larger in duration. This finding is in accord with a similar result from frog myelinated nerve fiber (Tasaki and Fujita, '48), although there is evidence to the contrary also from frog myelinated nerve fiber (Schoepfli and Erlanger, '41) and from the squid giant axon (Hodgkin and Katz, '49). Actually, the spike voltage of the crustacean nerve fiber is not markedly reduced until the temperature approaches near blocking value. In fact at 10°C, (fig. 12), the action potential voltage is as large or slightly larger than at 18°–20°C. As has been suggested (Tasaki, '49) to explain the conflicting results with frog myelinated fiber the increase in voltage may be due in part at least to the increase in medium resistance with cold. But if a fiber leaks ions when cooled, as already postulated, especially sodium or calcium, then the spike amplitude should certainly decline (Hodgkin and Keynes, '56) as observed here. But it remains to be explained why the squid giant axon potential shows no such variation even at near freezing temperatures (Hodgkin and Katz, '49).

Crustacean motor axons are capable of conducting impulses at temperatures as low as 5°C or slightly less. The action potential is extinguished by cold in the larger, less oscillatory responding fiber such as the fast closer at 4°–5°C, and conduction disappears between 1°C and 3°C in the smaller, highly oscillatory firing fiber such as the opener or slow closer. Thus the crustacean motor axon is able to conduct impulses in the same temperature range, from 3°–25°C, as found for the squid giant axon (Hodgkin and Katz, '49) and also the frog single myelinated fiber (Tasaki and Fujita, '48; Tasaki, '49, '53; Schoepfli and Erlanger, '41; Hashimura and Wright, '58) although the low temperature limit at which total extinction of the action potential of the squid and frog fiber occurred has not been reported. However, at these low temperatures only the all or none response is lost, for the local potential, greatly enlarged in the cold fiber, may be elicited for some time at near zero degree temperatures. These effects are reversible provided the fiber is not maintained at blocking temperature. The threshold value for eliciting the local response, as well as the all or none, increases rapidly at these extremely cold temperatures. A similar type of response has been reported (Grundfest, '55) as obtainable from squid giant axons poisoned by certain drugs.

If the stimulating current is assumed to displace or reorient abruptly membrane molecules, opening areas into which sodium ions may suddenly flow (trap door mechanism) then at low temperatures this displacement becomes more difficult as might be the case if the surrounding area became extremely viscous and there could be no abrupt or sudden shift. The amount of displacement becomes proportional to the applied pressure which means the amplitude of the sodium influx as recorded by local depolarization varies directly with stimulus strength. Such a model scheme also explains the loss of the all or none, assuming particles can not ''pop off'' from bonded sites in cold membranes. D. C. stimulation of any strength probably causes some sodium ion influx which is rapidly brought to an equilibrium different from the resting state value by the extrusion mechanism. The membrane potential and membrane excitability are thereby held in a more or less stable state but at somewhat different values from the resting state. This explains the shape of the dual shock excitability curve seen with the oscillatory fiber. In the case of the non-oscillatory fiber, a leaky fiber (Wright, Coleman and Adelman, '55), during applied subliminal DC the extrusion mechanism is not up to the task of equalizing the large influx and sodium accumulates inside, potassium is displaced and lost to the outside and excitability continually decreases; that is, accommodation takes place. At low temperatures the extrusion mechanism of nerve fibers may become so sluggish that the interior accumulation of sodium is even greater accounting for the marked increase in accommodation. Indeed it has been shown that sodium does collect in frog nerve tissue at low temperatures (Shaw and Simon, '55). It has also been shown that fibers continually stimulated or simply allowed to age in saline solution not only accumulate sodium (Keynes, '50), but lose the ability to fire repetitively and acquire increased accommodation (Wright and Adelman, '54; Wright, Coleman and Adelman, '55). At the same time there is probably potassium loss through leakage or displacement and this, too, must take part in producing the overall effect (Wright, Coleman and Adelman, '55).

If these arguments are correct it becomes apparent that the local response is a depolarization in itself directly associated with the inward flow of sodium ions. When transformed from an all-or-none to a local response the amplitude must then be limited to a voltage somewhat less than the sodium potential value, taking into account the accumulation of sodium ions in the fiber (Hodgkin and Keynes, '56). In other words, when initiated during conduction block, no matter how strong the applied stimulating voltage, the nerve response should not exceed the original spike height which we know to be determined by the sodium potential (Hodgkin and Huxley, '52).

The results of a typical experiment to investigate this point are illustrated in figure 17. The maximum size of the local response was determined using a fast closer fiber. The upper two pictures show the response obtained at the cathode at

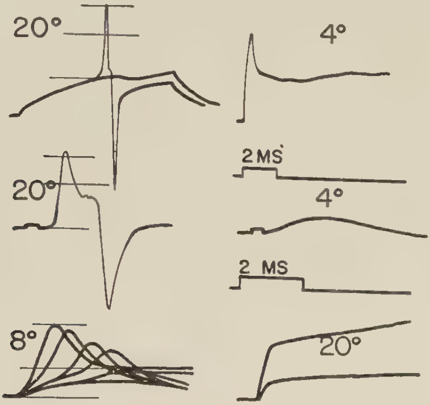

Fig. 17 Action potentials recorded from a fast closer fiber at 20°C and 4°C as indicated. Upper pictures fiber in oil recorded with strong D.C. stimulus at cathode. Slow sweep 2 millisecond calibration just below 4°C trace. Middle pictures recorded with fiber partly immersed, conduction distance 8 millimeters, fast sweep calibration below 4°C trace. Lower pictures at 8°C, fiber in oil, recorded at cathode with weak to strong D.C. stimulus (left picture) and artifact from dead fiber after four hours at 4°C (right picture).

20°C (left) and at 4°C (right). The middle two pictures show the conducted response obtained with a fast sweep at 20°C (left) and 4°C (right). The lower left picture shows the cathode response elicited by weak to strong currents after slight rewarming to 8°C, and the response from a dead fiber held at 4°C for four hours is shown in the right picture. These pictures are at fast sweep speeds also. In the 8°C picture the horizontal lines drawn designate the peak, the D.C. current artifact and zero current baseline respectively. Certainly the deflection above the D.C. current is less than the upward spike deflection at 20°C, and even if the D.C. current is included the overall deflection at 8°C is less than the spike. It is never greater than the spike, usually slightly less as expected.

In conclusion then it seems likely that the loss of excitability and the all or none response at low temperatures is due in part at least to the upset of the ionic concentration balance because of an influx of sodium, an outflux of potassium, or both. The actual changes of ionic fluxes and concentrations in these nerve fibers brought about by temperature changes have yet to be determined. Also, the effect of different ion concentrations on fibers subjected to cold, or heat, is not known and these ionic concentration ratios may well have something to do with the specific temperature range at which the nerves of different species function. Finally, it has been implied that the local response itself may be a sodium potential. This implication has been investigated and some rather interesting results have been reported elsewhere (Wright, '56).

SUMMARY

Single crustacean motor axons have been subjected to low temperatures and the changes in excitation and electrical properties studied with the following results:

1. Threshold value for D.C. stimulation is slightly reduced, for short duration stimuli markedly increased.
2. The chronaxie values are increased and the excitation rate constant k is decreased.
3. The accommodation time constant λ when measured by the exponential current is apparently markedly decreased but this is because of the change in k value. Actually, accommodation as shown by the dual shock method is very slow in onset and is moderately increased, meaning that the λ time constant value does decrease somewhat.
4. Fibers capable of repetitive firing continue to fire repetitively at low temperatures, but the frequency of the oscillation is reduced. This is because of the great lengthening of the refractory period with cold. The fact that repetitive

firing can be elicited at low temperatures substantiates the small decrease in λ value.

5. The recovery of excitability following an action potential is exponential with a markedly decreased rate constant at lower temperatures. This decay curve does not show any linearity at low temperatures.

6. The conduction velocity is reduced by a factor of 3 or 4 with a temperature reduction of twenty degrees in all fibers.

7. Conduction block occurs at 4°–5°C in the large non-oscillatory fiber types, 1°–3°C in the smaller oscillatory fiber type. The action potential spike maintains about the same voltage but increases in duration with mild cooling, decreases markedly in voltage and increases in duration at extremely low temperatures.

8. The sub-threshold local or initial response is enlarged both in voltage and duration with cooling. The decay of this potential elicited by brief shocks falls exponentially at normal temperatures with a rate constant of the approximate value given by the excitation theory equations. At low temperatures the decay is much slower and is better fitted by a linear equation.

9. When conduction block occurs, the all or none response is lost but the local response remains extremely enhanced in voltage. The maximum size depending on the stimulus strength is limited to the original spike size or slightly less. This suggests that sodium ions are responsible for the local as well as the spike potentials.

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THE REHYDRATION CONTRACTION OF FROZEN DRIED MUSCLE [1,2]

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SEVEN FIGURES

INTRODUCTION

During the course of some work that involved the extraction of muscle which had been rapidly frozen and dried, it was noticed that small fragments of the material would apparently contract when they were rehydrated. It was subsequently found that the same reaction had been observed by Hürthle ('09), who, while studying the cytology of insect wing muscles, noticed that the material which had been frozen and then dried over P_2O_5, contracted when rehydrated. Unfortunately, he only went as far as to observe that the muscle fibers relaxed slightly in glycerol and contracted when placed in dilute salt solutions.

The fact that such a preparation shortens when rehydrated is not sufficient grounds for assuming that the shortening is fundamentally the same process as the contraction of living muscle. Therefore, the shortening which takes place after rehydration has been studied under isometric, isotonic, and essentially unloaded conditions to learn to what extent it resembles muscular contraction. Perry ('50) has shown that the freezing and thawing of muscle considerably weaken the

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[2] A preliminary report of this work appeared in Fed. Proc., Vol. 14, 1955.

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contractile system as evidenced by the load that can be lifted by thaw rigor. The present technique offers an opportunity to observe the effects of freezing uncomplicated by thawing by substituting for it dehydration and rehydration. Furthermore, it was thought that such a preparation could be of general use in the study of muscle, provided that the contraction was similar to that of living muscle.

That the reaction is not simply an indifferent shortening of a dried linear protein is demonstrated by the fact that dead muscle which has been treated in the same way does not behave in a similar fashion, and that the normal dried muscle loses its ability to respond if it is exposed to room air for a few hours. Since muscles fatigued before drying shorten only slightly when rehydrated, it appears that the energy supply for this reaction is the same as the energy supply for living muscle. The present report is concerned only with describing the contraction that invariably occurs when dried fibers of the frog are immersed in dilute salt solutions and the subsequent shortening that can be produced by ATP.

MATERIAL AND METHODS

The *gracilis* and the *sartorius* muscles of the frog, *Rana pipiens,* were used; in a few of the earlier experiments the *gastrocnemius* was also employed. The muscles were isolated in Ringer's solution and securely fastened into an adjustable rigid frame which held them at resting length. The frame and the muscle were then rapidly plunged into liquid nitrogen. After fifteen minutes the muscles were removed and placed in a histological freeze-drying apparatus similar in design to that described by Glick ('52). Liquid nitrogen was used as a coolant for the traps. It is possible to dry small muscles over P_2O_5 or by immersion in cold acetone, but although muscles dried in this way will contract when rehydrated, their behavior is not very reproducible. The dried material was stored in evacuated tubes at — 15°C. Small samples were cut from the muscle as needed; these were kept in a desiccator except when bundles of fibers were actually being teased out for use. The frozen dried muscles showed no sign of shrinkage or collapse due to desiccation.

Measurements of the contraction of the free floating fibers were made by placing a few drops of the solution being tested on a bundle of three or four fibers, whose length had been determined, and measuring the final length after the contraction was completed. The amount of contraction was expressed as per cent shortening (final length/initial length × 100). A minimum of five observations was made with each test solution and the arithmetic mean of the five values for per cent shortening was used in expressing the data.

Isotonic contractions were recorded with a smoked drum kymograph using fiber bundles about 0.5 cm long with a cross sectional area between 0.01 and 0.2 mm^2. After the thickness of a separated bundle of dry fibers had been measured with an ocular micrometer, it was fastened at one end with a small clamp to a balanced muscle lever while the other end was held in a stationary clamp. The free length of the fiber bundle between the clamps was measured and the preparation was then immersed as rapidly as possible in the rehydrating solution. The load per mm^2 of cross sectional area was calculated from the weight which was placed on the muscle lever pan and the measured thickness of the bundles. Two measurements were made at the most compact point of the bundle, one of the thickest and the other of the thinnest dimension at this point. The approximate cross sectional area was taken as 80% of the product of these two dimensions.

To record isometric contractions, the bundles were mounted in the same way as for the isotonic contractions except that the upper clamp was attached to a torsion spring upon which a mirror was mounted. A 10 meter optical lever was used for amplification. Recording was done with a photokymograph.

All solutions were made with water redistilled in a pyrex glass still. The sodium salt of adenosine triphosphate (ATP) (Sigma Chemical Company) was used. Several different lots of ATP were used in making up solutions, which were stored in the frozen state. A single solution was not used for more than a few days.

RESULTS

The Contraction of Free-floating Fibers. In the dry state the fibers appear as almost white, opaque cylinders. Shortening follows almost immediately upon wetting. The rehydrated fibers show most of the features seen in living fibers except for the air bubbles entrapped within the cells by the entering solution and for occasional breaks in the sarcolemma.

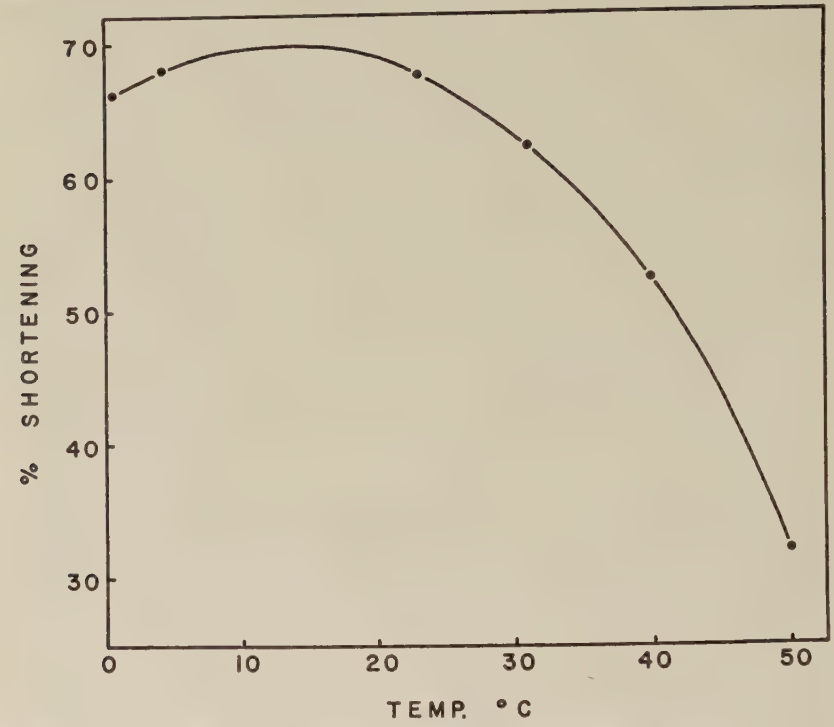

Fig. 1 The effects of temperature on the % shortening of fibers in 0.015 M KCl. Each point represents the mean of the % shortening of 5 fibers.

When exposed to distilled water, the free-floating fiber bundles shorten as much as 70% if the preparation is fresh. In those preparations that had been stored for one or two days, the shortening was between 60% and 70%. Since only water has been removed from the fibers and all of the ions are still present, it is not surprising that changes in the ionic strength and composition of the medium had very little effect. The effects of NaCl, KCl, $MgCl_2$ and $CaCl_2$ solutions were studied at ionic strengths (μ) from 0.01 to 1.0. With the exception of calcium, all the cations produced a shortening ranging from 50% to 70% in solutions of $\mu = 0.01$ to $\mu = 0.4$. In the case of calcium, reproducible results were obtained only at ionic strengths from 0.01 to 0.02. In this range there was a 60% shortening, but at the higher strengths there was a very wide scattering of values and lack of reproducibility. Above $\mu = 0.5$ the shortening produced by the other solutions decreased. At ionic strengths of 1.0 shortening was 10% or less, and the fibers started to lengthen again as soon as the shortening had ceased.

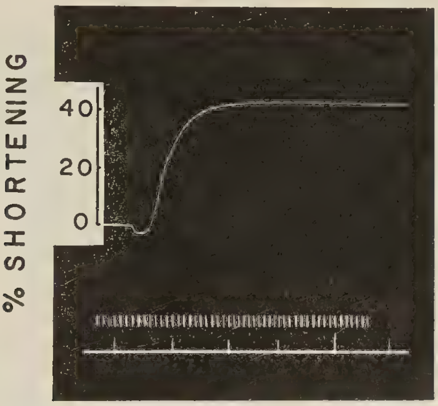

Fig. 2 Isotonic contraction in 0.15 M KCl. Figures represent % shortening. Load is 5.3 g/mm². Time signals: 1 second and 10 seconds.

The per cent shortening was studied in solutions of different pH using 0.05 M imidazole buffers. Over the range studied, pH 5.85 to 7.60, there was no effect. Changes in temperature affect the contractile behavior of frozen fibers as shown in figure 1. The optimum % shortening occurs between 5° and 20°C. The solution used was KCl at $\mu = 0.015$.

Muscles that had been narcotized with either isotonic KCl or $MgCl_2$ before freezing behaved in the same way as untreated muscles. The maximum shortening that occurred in muscles that had been fatigued before freezing was 20 per cent.

Isotonic Contractions. Figure 2 is a typical recording of the isotonic contraction that occurs when a bundle of fibers is immersed in distilled water or a dilute salt solution. Immediately upon wetting, the fibers lengthen slightly and then shorten rapidly. At room temperature the shortening is usually complete within 10 to 20 seconds, depending on the load being lifted. The % shortening and rate of contraction are not significantly altered by changes in the ionic strength between 0 and 0.15.

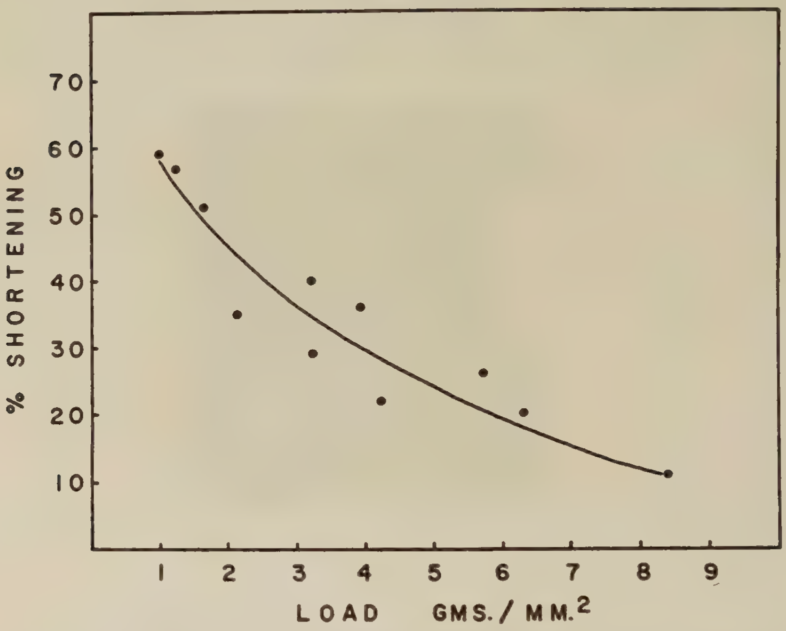

Fig. 3 Effect of load lifted, expressed in g/mm^2 of cross sectional area, upon the % shortening of fibers in 0.015 M KCl. Each point represents a single determination.

The per cent shortening in 0.015 M KCl as a function of the load in g/mm^2 of cross sectional area is graphed in figure 3. In 0.015 M KCl, the points fall approximately on a line between the values for the free-floating material which contracted between 60% and 70% with zero load and the value for the isometric determination which developed an average tension of 9.2 g/mm^2 with zero contraction. The relaxation rate of these frozen dried muscle fibers is a function of the load that they are supporting. With a light load, there is little or no relaxation, but as the load is increased the rate of relaxation increases (fig. 4).

Fibers in the shortened or contracted state are sufficiently elastic that upon being rapidly stretched to their original length and released, they immediately return almost to their contracted length. However, if shortening is prevented mechanically for the first 20 to 30 seconds after wetting, then little or no shortening takes place when the muscle is released. Similarly, if the fibers in the contracted state are stretched and held for 30 seconds, they will only shorten again very slightly.

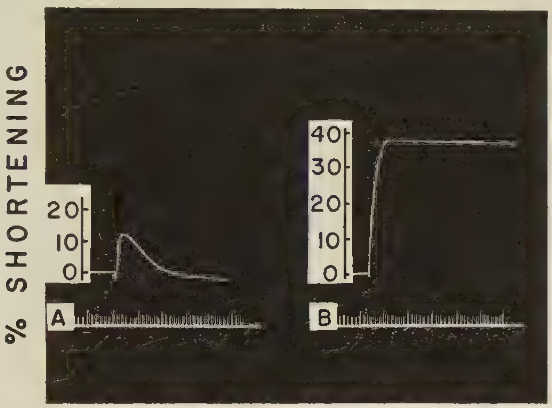

Fig. 4 Effect of load on the relaxation of fibers in 0.15 M KCl: (A) load of 7.5 g/mm^2; (B) 2.1 g/mm^2. Time marks: 10 seconds.

It is of interest to note that at any time during the normal course of contraction or relaxation, it is possible to immobilize the fibers by immersing them in liquid nitrogen. Upon being thawed out in the same solution in which the contraction was started, the contraction or relaxation continues practically without modification.

An attempt was made to find a solvent to which the fibers could be exposed at room temperature without either producing contraction or preventing contraction when the fibers were later immersed in dilute salt solutions. Diethyl ether, chloroform, acetone, or ethanol produce no contraction, but on subsequent exposure of the fiber treated with these solvents to KCl solutions there is no contraction. Apparently the contractile machinery has been completely destroyed or fixed by the solvent. However, it is possible to expose the fibers to petroleum ether for indefinite periods of time and have them contract normally when they are immersed in water or KCl solutions.

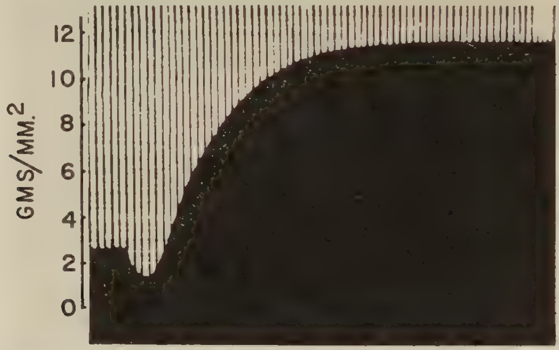

Fig. 5 Isometric contraction in 0.015 M KCl. One line every 0.25 seconds. Maximum tension developed 11.5 g/mm^2.

Isometric Contractions. Figure 5 is a recording of an isometric response of a bundle of dry fibers upon immersion in 0.015 M KCl; the same kind of a record is obtained with distilled water or other dilute salt solutions. The initial tension to which the fibers were subjected before wetting was about 200 mg. Immediately upon wetting there was a transient decrease in tension that lasted about one second, followed by an increase in tension that was complete by 10 to 15 seconds after the initial wetting. The maximum tension developed was 9.2 ± 2.89 g/mm^2 in 0.015 M KCl, and 12.3 ± 3.31 g/mm^2 in 0.10 M KCl. No significant difference could be found in the tension developed in g/mm^2 with changes in the ionic strength up to 0.15, and the type of cation used seemed to have very little effect.

Effects of ATP. The effects of ATP were investigated only to see whether the frozen-dried muscle behaved in this respect like glycerinated muscle or like living muscle. ATP produced an isotonic contraction in fibers that had previously contracted in KCl solutions (fig. 6). In contrast to glycerinated preparations (Bozler, '52 a), the frozen-dried preparations did not contract in the presence of 5 mM ATP to any great extent. Only when the rather unphysiological level of 20 mM ATP was reached was there a marked response, and even under these conditions the contraction was less than 50% of that produced by the initial wetting with an equal load. Under isometric conditions, the fibers showed a similarly low sensitivity to ATP. The response to ATP further differed from that of the glycerinated preparation in that variations of ionic strength, pH (from 6.0—7.5), and the presence or

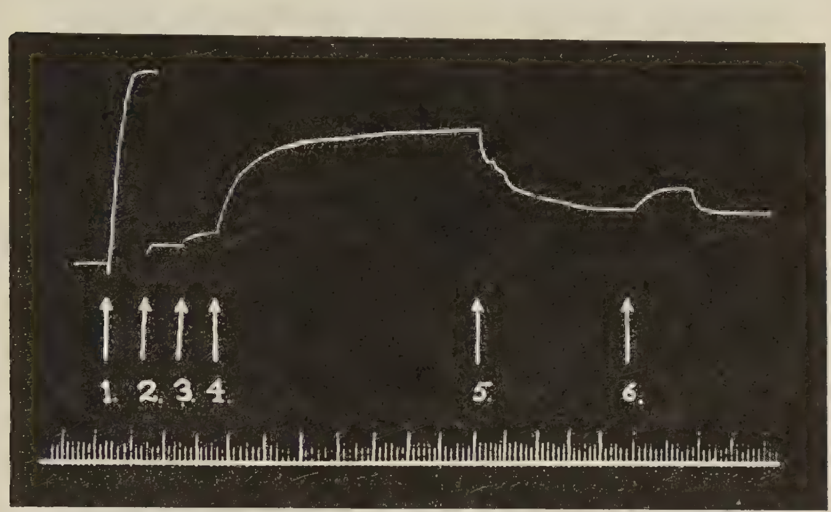

Fig. 6 Isotonic contractions produced by ATP: (1) dry fibers immersed in 0.15 M KCl (2) pulled back to approximately rest length (3) 5 mM ATP in 0.01 M $MgCl_2$ (4) 20 mM ATP in 0.01 M $MgCl_2$ (5) stretched and soaked in 0.15 M KCl again (6) 20 mM ATP in 0.01 $MgCl_2$ again. Initial contraction 28%. Load is 5.3 g/mm².

absence of Ca or Mg had no effect; repeated attempts to find a solution which would produce relaxation met with failure.

The only treatment of the many tried that would increase the response of the dry fibers to 5 mM ATP was exposure to 50% glycerol. When the dry fibers were immersed in 50% glycerol, the rate of tension development and shortening was much slower than in KCl; but after washing with KCl, exposure to 5 mM ATP caused a marked shortening and exposure to 20 mM ATP caused as much shortening as was produced by the initial exposure to KCl (fig. 7).

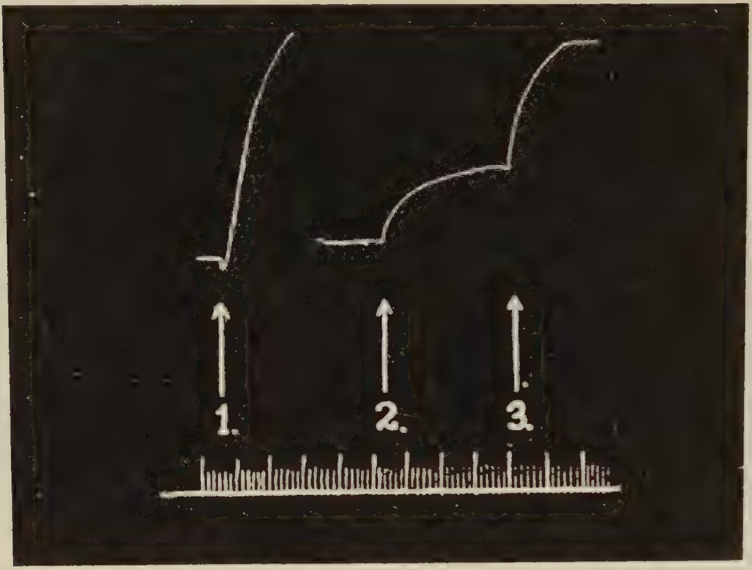

Fig. 7 Effect of 50% glycerol on the isotonic response to 5 mM and 20 mM ATP: (1) fiber immersed in 50% glycerol (2) immersed in 5 mM ATP after having been stretched and soaked in KCl (3) immersed in 20 mM ATP.

DISCUSSION

The average per cent shortening of the free-floating frozen dried muscle fibers corresponds very closely to the maximum shortening of 60% of living striated muscle. Both the isotonic and isometric contractions are also similar to those in living muscle, in spite of the large errors involved in measuring the cross sectional area of such irregularly shaped bundles of fibers. The average tension of 9 g/mm^2 that is developed by dried fibers falls well within the range of living frog *sartorius*, which develops a tension of from 8 to 15 g/mm^2, but well below the tension of 35 g/mm^2 reported for single living muscle fibers by Ramsey and Street ('40). When the effects of temperature on the per cent shortening of the dried muscle are compared to similar determinations made by Hadju ('50) on living frog muscle, it is seen that the two curves are similar but that the living material is more sensitive. At 25°C both preparations shorten the same amount, but with increasing temperature the extent of shortening of the living muscle decreases faster than that of the dried muscle. At 32°C Hadju's preparation shortened 45%, while the dried muscle was not inhibited to this extent until the temperature reached 42°C.

It appears that with regard to shortening and tension development the frozen dried muscle closely resembles living muscle. The freezing, dehydration and rehydration have not seriously damaged the contractile machinery; the system responsible for the contraction of living muscle is in these respects behaving as it does in living muscle. The contraction resulting from rehydration is not the same sort of process Bozler ('55 b) described that occurs when glycerinated fibers are exposed to distilled water, which develops a tension of only 5 g/mm^2. Furthermore the contraction of dried muscle does not resemble *rigor mortis* in that, according to Bendall ('51), one hour is required for *rigor* to develop at 37°C in the rabbit psoas with a maximum work load of 15 g cm/g of muscle, whereas the dried muscle develops full tension in 10 to 15 seconds and has a maximum working capacity of approximately 100 g cm/g. The most obvious difference between the contraction of dried muscle and living muscle is that the rate of shortening and tension development in the dried muscle is as low as that in the glycerinated preparations, which is extremely low in comparison with living striated muscle. A further difference between the contraction of living muscle and that of dried muscle is that in the latter the contraction is not followed by relaxation. In contrast to living muscle, a considerable load is required in order to stretch the fibers back to their original length (fig. 4).

The dried muscle differs from the glycerinated muscle most noticeably in that it lacks the sensitivity to ATP seen in the glycerinated preparations. Although the response to ATP is poor in comparison to the response of the glycerinated muscle, it does not follow that dried muscle is behaving less like living muscle. Evidence based on the injection of ATP into living muscle fibers is somewhat contradictory, due possibly to the presence of impurities in the solutions. However, the work of Falk and Gerard ('54) and Wiercinski ('56), indicates that the living contractile system does not respond to the presence of ATP. Furthermore Mommaerts ('55) has demonstrated that ATP is not used in the process of a single twitch of living muscle. Thus it is possible that the low sensitivity of the dried material to ATP is further evidence of its close physiological resemblance to living muscle, and that treatment with 50% glycerol alters it so that it shows the high sensitivity of glycerinated muscle.

It would be of interest to know at what point, between the time of freezing and the onset of the contraction that occurs when the fibers are rehydrated, the contractile machinery is activated. As Hürthle ('31) has demonstrated, a frozen muscle is a contracted muscle. It might be argued that the contraction which takes place on rehydration is simply a result of softening the rigid dry fibers so that they can complete the contraction which was initiated at the time of freezing. There are two points which suggest that such a simple explanation is not satisfactory. In the first place, immediately upon being rehydrated the fibers relax slightly (figs. 2 and 5). It is possible that this is a mechanical artifact, but it consistently occurred in spite of all attempts to eliminate it and it appeared to be roughly proportional to the load upon the fibers. Such a relaxation indicates that the fibers first become plastic and stretch before contraction starts. If contraction represented the release of fibers that were under tension in the dry state, then contraction would start as soon as they became plastic. In the second place, if the contraction which occurs on freezing is eliminated by KCl or $MgCl_2$ anesthesia, the fibers will contract when rehydrated just as do those from muscles which have not been anesthetized. Thus the contraction which takes place on rehydration is not dependent upon the muscle being in a contracted state during freezing. The most probable time for the activation of the contractile mechanism is during rehydration by the direct effect of the entering solution. The initially high ionic strength during the early phase of rehydration may decompose lipoprotein complexes allowing calcium to combine with the contractile system which would, as has been demonstrated by Heilbrunn ('40), lead to contraction.

SUMMARY

1. The contraction which results when small bundles of muscle fibers that have been frozen-dried are rehydrated, has been studied.

2. The amount of shortening of the frozen-dried fibers, their ability to do work, the maximum tension that they can develop, and the effect of temperature on these processes all demonstrate that the contractile system of living muscle is not destroyed by freezing and drying: the contraction produced by the rehydration of frozen-dried muscle is much like that of living muscle, except that the rate of contraction is much slower.

3. The rehydrated fibers will respond to ATP only at high concentrations and are insensitive to changes in pH and ionic composition of the medium; the sensitivity to ATP can be increased by previous exposure to 50% glycerol.

ADDENDUM

Since the acceptance of this article for publication in June, 1957, two articles on closely related subjects have appeared. Mueller and Szent-Györgyi (Science, *126:* 970-71, 1957) have confirmed the findings reported here and in a previous note by the author (Anat. Rec., *124:* 307, 1956) on the ability of the contractile system to withstand drying in cold acetone. Hunt and Matheson (Nature, *181:* 472-3, 1958) in studying the effects of freeze-drying on beef and fish muscle did not report any contraction on rehydration, but did find that the fibers contracted when exposed to ATP. The absence of a rehydration contraction may well have been due to warming the muscles before they were completely dry.

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POTASSIUM EXCHANGE IN HUMAN ERYTHROCYTES

I. GENERAL ASPECTS OF THE FLUORIDE EFFECT

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INTRODUCTION

Wilbrandt ('37, '40) first described the loss of potassium and water, and the increased resistance to osmotic hemolysis of human red cells incubated at 37°C in the presence of NaF. The effect of K loss was confirmed by Davson ('41), Harris ('41), Danowski ('41), Ponder ('49), Green and Parpart ('50, '53), Dunker and Passow ('50), Maizels ('51), and Taylor et al. ('52). Davson, and Green and Parpart studied rabbit cells; the others used human cells.

Wilbrandt and Davson both reported the striking influence of NaF concentration upon K loss, though Ponder was unable to confirm this effect. K loss is small at low (0.005 M) and high (0.120 M) concentrations, but extremely rapid at intermediate (0.02 M–0.06 M) concentrations.

In 1950, Sheppard and Martin, and Raker et al. found the rate of K exchange between red cells and their medium to be about 1.6 μM/ml of cells/hr. From this, it could be inferred that the complete arrest of K uptake by the cells due to the inhibition of glucolysis by NaF could only account for a maximum loss of 1.6 μMK/ml cells/hr. This is approximately the net K loss Maizels observed in the presence of 0.004 MNaF, and in a more recent publication (Maizels, '54), has been interpreted as due to failure of K uptake in the presence of

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a normal outgo rate. At NaF concentrations of 0.02 M–0.06 M, however, net K loss exceeds 1.6 μM/ml of cells/hr. and may reach 30 μM/ml of cells/hr. This net loss so far exceeds the outgo in normal cells that an additional effect of incubation with NaF to increase the outgo rate has been apparent to all investigators. This has been ascribed to a change in the permeability of the membrane resulting from the incubation. Wilbrandt suggested that the permeability change was secondary to a diversion of metabolism away from the normal glucolytic pathway. The slowing of K loss at high NaF concentrations he thought due to inhibition of the alternate pathway. According to this scheme, the excessive K loss which occurred at intermediate NaF concentrations would also be diminished. He found that added fructose, phosphoglycerate, phosphopyruvate, pyruvate, cyanide, and methylene blue all inhibited the fluoride effect. Hexose diphosphate exaggerated the fluoride effect. Davson found that sulfite, oxalate, citrate, and glycerol phosphate also diminished the NaF effect, while lactate increased the effect.

The experiments reported in this and the succeeding paper were undertaken to study the separate inward and outward rates of K movement with the aid of K^{42} at various NaF concentrations and to study the effect of pyruvate on the NaF effect. A reasonably accurate measure of K movement in the preparation has resulted: low concentrations of NaF (0.005 M) inhibit the uptake of K, leaving outgo unaffected. The cell K is thus almost completely isolated from the K of the extracellular fluid (ECF). Intermediate concentrations (0.025 M) have a second effect: a fraction of cell K at first isolated from the ECF K begins to exchange almost immeasurably fast with the ECF K. The size of this fraction of cell K increases with time. The net movement of K during this rapid exchange is with the concentration gradient (usually outward). At high MaF concentrations, a third effect is seen. The development of a rapidly exchanging fraction of cell K is itself inhibited and the K loss from these cells is correspondingly small. Added pyruvate restores exchange towards normal in the presence of 0.005 M NaF, slows the formation of the rapidly exchanging fraction of cell K at intermediate NaF concentrations, and is without effect at high NaF concentrations. Preliminary reports of this work have been published (Eckel, '53, '54).

METHODS

Preparation of red cell suspension media: The usual incubation medium contained 10 mM Na_2HPO_4 (adjusted to pH 7.4 with HCl), 135 mM NaCl + NaF, 5 mM KCl, and 2.0 gm of glucose per liter. No Ca or Mg salts were used. In experiments done in the presence of 0.120 M NaF, fluoride salts replaced chloride salts in the above medium during washing as well as during incubation. In experiments done in the presence of 0.005 M and 0.025 M NaF, the cells were washed in a medium containing 5 mM and 25 mM less of NaCl per liter, respectively. This deficit was made up by the addition of NaF to experimental flasks and of NaCl to control flasks in a volume equal to 10% of that of the red cell suspension at the beginning of the incubation. Sodium pyruvate addition (0.003–0.010 M) resulted in an increase in the salt content of the incubation medium. The salt concentration of all flasks in a given experiment were equal, however; control vessels received equivalent amounts of NaCl. Na salts were replaced in part or entirely by K salts in some experiments.

Experimental Procedure: Blood was taken from the antecubital vein of healthy medical students and laboratory personnel in syringes wet with heparin. In most experiments, 60 ml from each of two donors of the same ABO and Rh type were pooled for use in a single experiment. This blood was washed twice with one of the washing media previously chilled to 5°C, and the volume was adjusted to slightly more than the original blood volume by removal of the supernatant medium. The cells, after resuspension, were placed in Erlenmeyer flasks plugged with cotton which were shaken in a constant temperature bath at 38°C. After 10 minutes allowed for temperature equilibration, the experiment was begun, and this time is referred to below as the zero time of the experiment even for experiments in which 0.120 M NaF was present during washing of the cells. Zero time was usually about 40 minutes after the venesection.

At zero time, NaF and other substances under study were added to the flasks. $K^{42}CO_3$, appropriately diluted in the medium in use, was added in 50–100 µl amounts to the flasks at the times indicated in individual experiments. After the additions, the flask contents mixed for 30 seconds prior to sampling. Samples of the medium were obtained by centrifugation of 4 ml aliquots of the suspension for two minutes in a table model angle head centrifuge. The indicated time of each sample in the data is two minutes after the beginning of centrifugation and about 2¼ minutes after removal of the sample from the flask. At this time, the centrifuge was turned off. The time for the head to come to rest and for the clear supernatant to be removed was disregarded.

The hematocrit was measured in Wintrobe tubes after spinning at 2200 g. for 20 minutes. The accuracy of the hematocrit was checked by measuring the recovery of K^{42} added to the medium of cell suspensions containing 0.005 M and 0.120 M NaF which reduce K^{42} entrance into the cells. These data showed that the observed hematocrit was no more than 103% of the true hematocrit. This was a satisfactory estimate for most of the work presented, and all hematocrit data presented are observed values. The significance of the probable error in the hematocrit is discussed under results. Counting of the medium and blood was done on 1 ml samples in an end-window counter at rates of 4000–10,000 cpm. K and Na were determined by means of the Beckman flame photometer attachment to the model DU spectrophotometer. Chloride was measured directly on dilute samples of the medium and on tungstic acid filtrates of the red cell suspensions by an ultra-micro modifiication of the mercurimetric titration of Schales and Schales ('41) described previously (Eckel and Smith, '53). Glucose was measured by the technic of Nelson ('44) and Somogyi ('45) filtrates of blood. Lactate was measured by the technic of Barker and Summerson ('41), using the precautions described by Miller and Muntz ('38) to avoid interference by pyruvate. Utilization and production are calculated by the change in glucose and lactate in the suspension, expressed per ml of cells.

Kinetic analysis: Red cell systems have been analyzed as ideal two compartment systems. Though there is evidence of a small inhomogeneity in the cell K (Solomon and Gold, '55), the two compartment model has nevertheless been quite satisfactory in this work. In a suspension poisoned with NaF, the cell volume and total cell K both decrease, the medium or extracellular fluid (ECF) volume and K increase, the inward and outward exchange rates change, and in some cases, good evidence of a three compartment system appears. The analysis, below is based upon the formulations of Sheppard and Martin ('50).[2]

The two compartment system; unequal inward and outward rates: This is the simplest situation met in fluoride poisoned cells: though K enters the cells, the outward migration of K exceeds the inward. Total cell K decreases and ECF K increases with or without change in the volume of the compartments.

The following terms are used as defined by Sheppard and Martin.[3]

$S =$ total amount of K per ml of red cell suspension
$S_1 =$ total amount of K in the ECF per ml of red cell suspension
$S_2 =$ total amount of K in the cells per ml of red cell suspension
$dS_{21} =$ amount of K transported from the ECF to the cells of 1 ml of red cell suspension in time dt
$dS_{12} =$ amount of K transported from the cells to the ECF of 1 ml of red cell suspension in time dt
$dS_2 =$ net change in S_2 due to inequality of opposing transfers
$R =$ total radioactivity per ml of red cell suspension
$R_1 =$ total radioactivity in the ECF per ml of red cell suspension

[2] It is a pleasure to acknowledge the help of Dr. W. J. MacIntyre of this institution and of Dr. I. M. Krieger of Case Institute of Technology in preparing this analysis.

[3] It should be emphasized that S and R refer to total amounts of K and radioactivity, respectively, in a sample. In this case, the sample size is 1 ml. S and R are not concentrations.

R_2 = total radioactivity in the cells per ml of red cell suspension
a_1a_2 = specific activity of K in the ECF and cell, respectively
$dS_{21}/dt = \rho_1.\rho_1$ is the rate of K entrance into the cells of 1 ml of red cell suspension
$dS_{12}/dt = \rho_2.\rho_2$ is the rate of K exit from the cells of 1 ml of red cell suspension

The initial steps of the Sheppard and Martin derivation are repeated, yielding

$$\frac{da_1}{(a_1S - R)\ dt} = \frac{-\rho_2}{S_1S_2}$$

Since

$$\frac{da_1}{(a_1S - R)\ dt} = \frac{1}{S}\ \frac{d\ \ln\ (a_1S - R)}{dt},$$

these useful relations are obtained:

$$\frac{d\ \ln\ (a_1S - R)}{dt} = \frac{-S}{S_1S_2}\ \rho_2 \qquad \text{Eq. (1)}$$

$$\frac{d\ \ln\ (a_2S - R)}{dt} = \frac{-S}{S_1S_2}\ \rho_1 \qquad \text{Eq. (2)}$$

In practice, the function $\ln\ (a_1S - R)$ (or $\ln\ [R - a_1S]$) is plotted without regard for sign against time as the abscissa. The slope of the line, determined graphically at time t is $d\ \ln\ (a_1S - R)/dt$. This value, with the measured values of S, S_1, and S_2 permit the calculation of ρ_2. ρ_1, the inward rate, may be calculated similarly by equation 2.

ρ_1 may be calculated in two other ways. The difference between ρ_1 and ρ_2 is the net rate of cell K loss ($-dS_1/dt$) and is determined graphically by plotting S_1 against time. Therefore,

$$\rho_1 = \rho_2 - dS_1/dt \qquad \text{Eq. (3)}$$

For the interval immediately following the addtion of K^{42} when a_2 is very small and may be neglected, the inward rate may be calculated by means of the equation:

$$\rho_1 = \frac{-dR_1/dt}{a_1} \qquad \text{Eq. (4)}$$

The term dR_1/dt may be replaced by $\Delta R_1/\Delta t$ for short intervals. When the uptake of K^{42} is inhibited, as occurs in many of the experiments reported, a_2 remains so low throughout the experiment that equation 4 actually furnishes a useful estimate of ρ_1 throughout the experiment.

The relationships shown in equations 1–4 permit the calculation of unequal and changing exchange rates. They require only that all the K in the system be in two uniformly mixed pools. This assumption was tested in the present experiments by the addition of K^{42} at zero time to one flask and at an interval after zero time (usually three hours) to a duplicate flask incubated simultaneously. The exchange rates were then calculated. In practice, net K loss was sometimes larger in the flask from which samples were obtained repeatedly up to three hours than in the flask incubated undisturbed during this time. This may have been due to the mechanical trauma of the pipetting, or to the increased surface — volume ratio of this flask as its volume decreased. When the exchange rates calculated for the two flasks were identical for the interval after addition of the K^{42} to the second flask, it was concluded that the cell K was behaving as a homogeneous pool. Such evidence was obtained in control incubation flasks without added NaF. The existence of a fraction of cell K exchanging more slowly than that which has exchanged in the three hour interval is not excluded, but a more rapidly exchanging fraction is excluded. The acceptance of red cell K as a homogeneous pool of K in these flasks is based upon this evidence and upon the results of Sheppard and Martin ('50) and Raker et al. ('50), in a diluted plasma medium.

The demonstration of two cellular fractions of cell K: In the presence of 0.025 M and 0.120 M NaF, the entrance of K^{42} added at zero time virtually stops after two–three hours equilibration. This was shown to be due to complete equilibration of the K^{42} with a fraction of cell K, since K^{42} added to a second identical flask at three hours entered the cells very rapidly and to exactly the same extent within ½ hour as had occurred in the full 3½ hours in the first flask. The two fractions of cell K are denoted by two additional symbols and the amount of K with which K^{42} equilibrated is determined by isotope dilution:

S_D = rapidly exchanging cell K
S_R = residual, slowly or non-exchangeable cell K

Hence,

$$S_2 = S_D + S_R,$$

and

$$S_D = \frac{R}{a_1} - S_1 \qquad \text{Eq. (5)}$$

The variation in size of S_D under different conditions is thus easily measured.

Presentation of results: The hematocrit (hct.), total K per ml of suspension (S), the total radioactivity per ml of suspension (R), the counts per ml of ECF (N_1) and the K concentration per ml of ECF ($[K]_1$) are measured. S_1, R_1, S_2, R_2, a_1, and a_2 are calculated:

$$S_1 = [K]_1 \ (1\text{-hct.})$$
$$R_1 = N_1 \ (1\text{-hct.})$$
$$S_2 = S - S_1$$
$$R_2 = R - R_1$$
$$a_1 = \frac{N_1}{[K]_1}$$
$$a_2 = \frac{R_2}{S_2}$$

The concentration of K in the cells $[K]_2$ is expressed per liter of cells (S_2/hct.) or per liter of cell water. The cell water is estimated as 71% of the initial hematocrit (hct_o) (Peters, '35). Subsequent changes in hematocrit were assumed to be due to changes in cell water. The calculation is as follows:

$$\text{cell water} = hct_t - 0.29 hct_o$$

The inward and outward exchange rates (K_{in} and K_{out}) in μM K/ml of cells/hr. calculated on the basis of the original cell volume, since considerable decrease in cell volume occurs in some experiments:

$$K_{in} = \rho_1/\text{hct.}$$
$$K_{out} = \rho_2/\text{hct.}$$

RESULTS

The exchange of K in phosphate buffered saline: Human red cells incubated in the standard medium with or without

0.01 M pyruvate for periods up to 4 hours neither gain nor lose significant amounts of K or water. K^{42} added at zero time, or at some subsequent time up to three hours after the beginning of the experiment, disappears in a regular fashion (fig. 1). Exchange rates for normal cells with or without pyruvate vary between 1.7 and 2.2 μM per ml packed cells per hour.

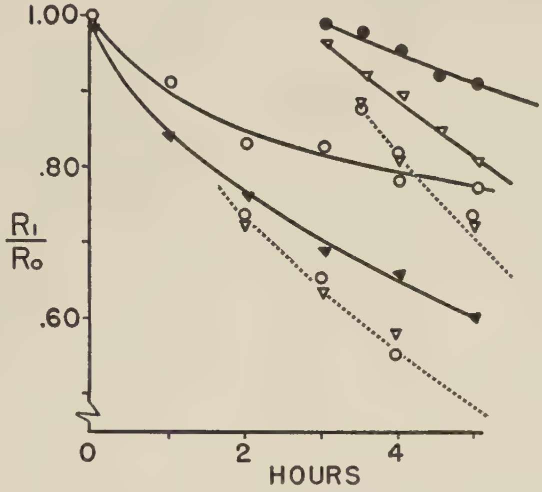

Fig. 1 Fraction of K^{42} remaining in medium of human red cell suspension (ordinate) as a function of time of incubation at 38°C (abscissa). Solid lines from experiment V 117. Circles: 0.005 M NaF alone. Triangles: 0.005 M NaF + 0.0010 M pyruvate. Dotted lines from comparable control experiment. Triangles: control flask. Circles: control + 0.010 M pyruvate. In this and succeeding figures, curves starting after zero time represent experiments in which K^{42} was added at the indicated interval to separate flasks identical to those in which K^{42} was added at zero time.

The influence of NaF upon K loss: As described originally by Wilbrandt, the effect of NaF depends upon the concentration of NaF used for the incubation. Preliminary data shows that K loss in the experimental period is greatest at NaF concentrations of 0.025 M–0.06 M and is quite small at very low or very high NaF concentrations. These results led us to investigate K exchange at three NaF concentrations which, it was hoped, would be representative of the effects of NaF over the spectrum of concentrations shown in table 1. At the same time, the modification of the fluoride effect by pyruvate was studied.

K exchange in the presence of 0.005 M NaF: A small steady net loss of K occurs, accompanied by a slight increase in cell water (table 1). K^{42} enters the cells more slowly in the presence of fluoride than in its absence. The decrease in rate is partially corrected by pyruvate (fig. 1). The exchange rates may be obtained in a variety of ways using the derived data in table 1, as indicated in the discussion of kinetics. All methods of calculation are quite consistent for the first three hours of incubation. The difference in rates late in the experiment using different methods of calculation are due to the error inherent in the hematocrit technique used. When the true hematocrit is assumed to be slightly smaller than the observed, the calculated values for net loss and K_{out} increase. The estimates of K_{in} according to equations 2 and 4 decrease, and that according to equation 3 increases, so that they agree with each other. The rates are about the same whether K^{42}

TABLE 1

Effect of 0.005M NaF on K exchange at 38°C

Experiment V-117

TIME	ECF K	L-HCT.	S_1S_2/S	$d \ln(R-a_1S)/dt$	$d \ln(R-a_2S)/dt$	dS_1/dt
hrs.	*mM/1*					
		Flask F:	$S = 33.5$	$R = 19846$	$hct_0 = .368$	
0	4.76	.632	2.74	.282	.120	.36
1	5.36	.630	3.04	.219	.094	.40
2	6.06	.628	3.38	.183	.068	.41
3	6.76	.625	3.69	.152	.052	.45
4	7.44	.620	3.97	.141	.036	.48
5	8.24	.625	4.36	.131	.032	.52
		Flask 3F:	$S = 35.7.$	$R = 22473.$	$hct_0 = .370$	
3	6.50	.626	3.60	.158	.048	.37
3.5	6.88	.624	3.77	.151	.047	.37
4	7.00	.622	3.82	.142	.037	.37
4.5	7.28	.625	3.97	.140	.030	.37
5	7.74	.622	4.15	.124	.029	.37

is added at zero time or at three hours (table 2). This indicates that the decreased rate of entry of K^{42} after three hours is due to a decrease of K entrance into a large fraction, or presumably, all of cell K. K_{out} is little affected by 0.005 M NaF. Other experiments (table 3) have yielded higher values for K_{in}. In one (experiment V96), a clear difference in exchange rates between flask 3 and 3F exists at three hours. This suggests one part of cell K in flask 3F exchanging more rapidly than a second fraction whose rate of exchange is simultaneously being measured in flask F. The discrepancy in K_{in} is small compared to that seen in the presence of 0.025 M NaF, but the occurrence of an initially rapid uptake rate which quickly slows, as seen in flask 3F is similar to the kinetics observed where a small rapidly exchanging fraction of cell K is present (Eckel, '58, fig. 1). The higher uptake rate in this experiment during the first two hours of incubation may be due to the higher K concentration in the medium, but other experiments have not shown a clear effect of extracellular K concentration on uptake.

TABLE 2

Calculated exchange rates of erythrocyte suspensions in the presence of 0.005M NaF

Experiment V-117

	Basis of calculation:	K_{in}			K_{out}	NET LOSS
FLASK	TIME	Eq. 2	Eq. 3	Eq. 4	Eq. 1	$-d S_1/dt$
F	0	+ 0.9	+ 1.1	+ 0.9	— 2.1	— 1.0
	1	0.8	0.7	0.7	1.8	1.1
	2	0.6	0.6	0.6	1.7	1.1
	3	0.5	0.3	0.4	1.5	1.2
	4	0.4	0.2	0.3	1.5	1.3
	5	0.4	0.1	0.2	1.6	1.4
3F	3	0.5	0.5	0.5	1.5	1.0
	3.5	0.5	0.5	0.5	1.5	1.0
	4	0.4	0.4	0.5	1.4	1.0
	4.5	0.5	0.5	0.6	1.5	1.0
	5	0.3	0.4	0.6	1.4	1.0

From these experiments it is concluded that 0.005 M NaF causes a progressive slowing in the rate of K uptake by the cell without any consistent effect upon the outgo rate. In one experiment, cell K divides into two fractions with differing exchange rates. From the data, neither the size of the two fractions nor their exchange rates can be calculated.

TABLE 3

K exchange rates of erythrocyte suspension in the presence of 0.005M NaF

FLASK	Basis of calculation: TIME	K_{in}	K_{out}	NET LOSS	ECF [K]
		Eq. 3	Eq. 1	$-dS_1/dt$	
		Experiment V–91			
F	0	1.1	1.4	0.3	5.14
	1	0.6	1.5	0.9	5.22
	2	0.5	1.6	1.1	5.76
	3	0.6	1.7	1.1	6.10
	4	0.7	1.8	1.1	6.58
3F	3	0.9	1.3	0.4	5.90
	3.5	0.9	1.3	0.4	6.34
	4	1.0	1.3	0.4	6.36
	5	1.0	1.4	0.4	6.52
		Experiment V–96			
F	0	1.6	3.0	1.6	15.7
	1	0.9	2.5	1.6	16.6
	2	0.9	2.5	1.6	17.5
	3	0.4	2.0	1.6	18.3
	4	0.3	2.0	1.6	19.2
3F	3	1.9	3.6	1.6	18.3
	4	1.6	3.2	1.6	19.2
	5	0.9	2.6	1.8	20.0
	6	0.4	2.2	1.8	20.9

If the more rapidly exchanging fraction exchanges as rapidly as that which occurs with 0.025 M NaF, as seems plausible, it is extremely small in size (Eckel, '56). No conclusion is made concerning the dependence of K uptake on the K concentration of the medium. The gradual slowing of K exchange resembles the slowing of Na exchange (Maizels, '51) and phosphate exchange (Mueller and Hastings, '51; Prankerd and Altman, '54) during incubation with NaF.

The effect of 0.01 M pyruvate in the presence of 0.005 M NaF: K exchange with and without pyruvate was studied in each experiment. The results of all are shown in table 4. Pyruvate partially restores the uptake rate. The outgo rate is consistently below that in control suspensions except in

TABLE 4

K exchange rates of erythrocyte suspensions in the presence of 0.005M NaF and 0.010 M pyruvate

Calculations as in table 3

FLASK	TIME	K_{in}	K_{out}	NET LOSS	ECF [K]
	hrs.				
		Experiment V–117			
FP	0	+ 1.4	— 2.3	— 0.9	4.92
	1	1.2	1.8	0.6	5.24
	2	1.2	1.5	0.3	5.72
	3	0.7	1.0	0.3	5.90
	4	0.7	0.9	0.2	5.96
	5	0.6	0.7	0.1	6.30
3FP	3	0.7	1.1	0.4	5.44
	3.5	0.7	1.1	0.4	5.52
	4	0.8	1.2	0.4	5.76
	4.5	0.8	1.2	0.4	5.76
	5	0.8	1.2	0.4	5.96
		Experiment V–91			
FP	0	1.0	1.6	0.6	4.86
	1	1.0	1.7	0.6	5.06
	2	1.1	1.7	0.6	5.24
	3	1.2	1.8	0.6	5.58
	4	1.2	1.8	0.6	5.66
3FP	3	1.3	1.5	0.2	5.58
	3.5	1.3	1.5	0.2	5.54
	4	1.3	1.5	0.2	5.58
	5	1.4	1.5	0.2	5.66
		Experiment V–96			
FP	0	1.0	1.2	0.2	4.97
	1	1.1	1.3	0.2	5.06
	2	1.1	1.4	0.2	5.12
	3	1.2	1.4	0.2	5.26
	4	1.2	1.4	0.2	5.40
3FP	3	1.0	1.3	0.3	4.98
	4	1.0	1.3	0.3	5.06
	5	1.1	1.4	0.3	5.48
	6	1.1	1.4	0.3	5.50

experiment V91. Net loss occurs in every experiment, but is quite small. Cell K remains homogeneous.

Glucose uptake in the presence of 0.005 M NaF: Five experiments were done on glucose consumption of red cell suspensions containing 0.005 M NaF with and without 0.010 M pyruvate. The results of all were uniform. No glucose is consumed in the presence of 0.005 M NaF. One experiment is shown in figure 2 (curve F). The disappearance of glucose or other reducing material during the first hour and its reappearance later is characteristic of all experiments. The effect of the further addition of sodium pyruvate on glucose consumption is less uniform. In two experiments, the glucose consumption equals that in the control flask (fig. 5, curve FP_1). In two, the control glucose consumption is maintained only for one hour after which no further glucose disappears (fig.

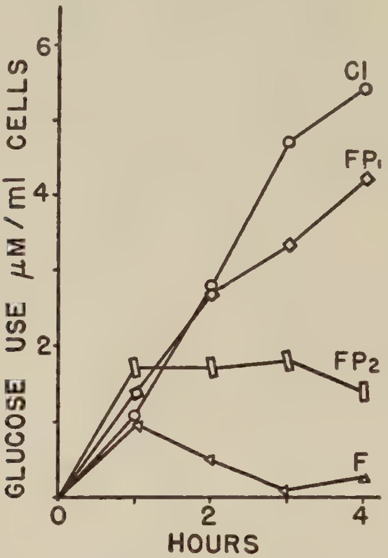

Fig. 2 Cumulative glucose disappearance from red cell suspensions (ordinate) incubated in presence of 0.005 M NaF (curve F), and 0.005 M NaF + 0.010 M pyruvate (curves FP_1, FP_2) and in control medium (curve Cl) as a function of the time of incubation (abscissa). The decrease in glucose utilized after one hour shown in curve F represents a reappearance of reducing material in the suspension.

5, curve FP_2). In one experiment, no glucose is consumed from the start.

The effect of 0.025 M NaF on K exchange: The presence of 0.025 M NaF has marked effects on the distribution of water, as well as K, between red cells and medium. The losses of K and of water per ml of cells in a 6 hour experiment are presented in figure 3.

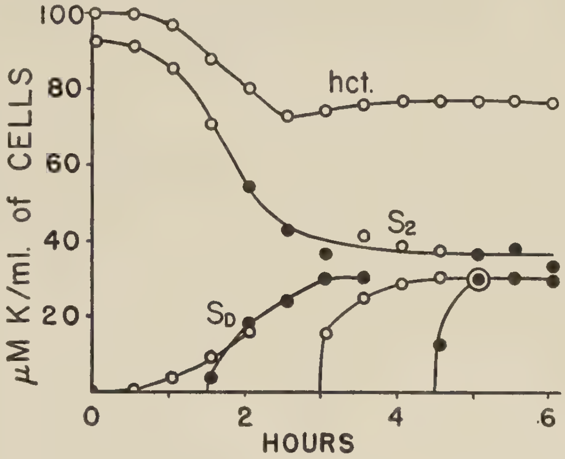

Fig. 3 Effect of incubation of human erythrocytes in presence of 0.025 M NaF on hematocrit (hct.), cell K (S_2), and rapidly exchanging cell K (S_D). Ordinate: hematocrit as per cent of initial hematocrit; S_2 and S_D in μM K per ml cells. Abscissa: time of incubation at 38°C. Open and closed circles represent analyses from separate flasks to which K^{42} was added at zero (open circles), 1½ (closed circles), 3 (open circles), and 4½ hours (closed circles).

These data show that after an initial lag period of about one hour there is a phase of extremely rapid K loss lasting one–two hours. The maximum net loss rate observed in this experiment occurs in the interval between 1½ and two hours when it was 39.5 μM K per ml cells per hour or 43% of the cell K per hour. As the medium K concentration rises, the rate of K loss falls until in many experiments it has essentially stopped in 4½ hours. In other experiments, such as that shown here, K loss continues for the duration of the experiment. The simultaneous loss of water indicated by the fall in hematocrit also is at a maximum at the time of maximum K loss, and then ceases abruptly in all experiments at about 2½ hours. Thereafter the hematocrit remains constant or increases slightly until the end of the experiment.

In the experiment shown in figure 3, K^{42} was added to each of the 4 flasks successively at 0, 1½, 3, and 4½ hours. The disappearance of the K^{42} from the medium is shown in figure 4. The initial rate of disappearance at zero time indicates an inward exchange rate of 0.5 M K/ml cells/hr. according to equation 4. The outward rate, according to equation 3 is 1.4 μM K/ml cells/hr. These rates are comparable to those seen with 0.005 M NaF after several hours incubation. From this it appears that the initial effect of 0.025 M NaF is identical to, but more intense than that of 0.005 M NaF. When K^{42} is added at any time after 1½ hours, the same amount of radioactivity enters the cell within a half hour as has entered since zero time in the first flask. From this it is concluded that sometime prior to 1½ hours, the K of red cells poisoned with this concentration of fluoride divides into two fractions. The first of these exchanges almost immeasurably fast with the medium K, and the second exchanges very slowly, if at all, with the medium K.

The size of the rapidly exchanging fraction, S_D, is calculated according to equation 5. The results of this calculation for this experiment are shown in figure 3. The size of this pool increases from 17 μM at two hours (30% of cell K at that time) to 30 μM after three hours (81% of cell K at that time). where S_D could be evaluated at three hours with a final medium K concentrations all close to the mean of 31.2 mM/1, S_D varied from 33–81% of cell K with an average of 56% of cell K. In 7 experiments in which S_D could be measured after 4½ hours incubation, the medium K concentration averaged 35.7 mM/1 and S_D 83% of cell K. The near arrest of K loss occurs when K is still unequally distributed between the cells and the medium. This apparent equilibrium is discussed further in the accompanying paper.

The effect of pyruvate in the presence of 0.025 M NaF: The general effect on cell K is unchanged. K is lost and a rapidly exchanging fraction appears (fig. 5). The fluoride effect is modified in two ways: (1) The rate of development of a rapidly exchanging K pool is slowed, so that at any given time, the size of S_D is about one third that present in the fluoride poisoned cells without pyruvate. (2) Corresponding to the diminution in S_D, there is a diminution in the total loss of K and water from the cells.

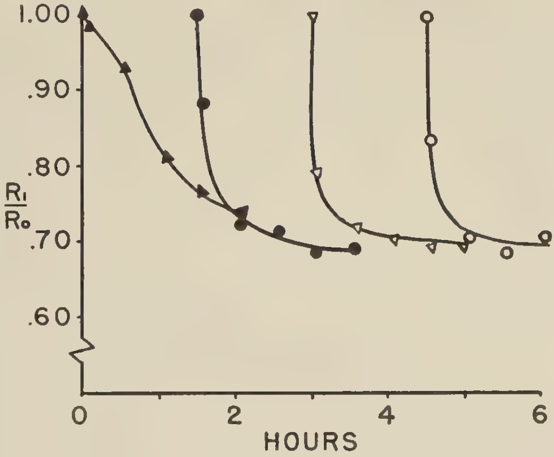

Fig. 4 K^{42} loss from medium of 4 identical flasks incubated simultaneously with 0.025 M NaF. Coordinates as in figure 1. Closed triangles: K^{42} added at zero time. Closed circles: K^{42} added at $1\frac{1}{2}$ hours. Open triangles: K^{42} added at three hours. Open circles: K^{42} added at $4\frac{1}{2}$ hours. Same experiment as that shown in figure 3.

Since the effect of pyruvate in the presence of low concentrations (0.005 M) of NaF is to restore K transport towards normal, it is of interest that during the first hours of incubation K transport appears nearly normal and there is little K loss. There is no indication that pyruvate in the presence of 0.025 M NaF leads to any K exchange between residual cell K and medium, however, once S_D is formed. The failure of the residual cell K to exchange is shown by the fact that all the K^{42} taken up by the cells during 3½ hours is shown to exchange rapidly with medium K if K^{42} is added at three hours. There is little, if any, difference in the rate of exchange of K^{42} between the medium and S_D as a result of the presence of pyruvate.

Metabolism of red cells poisoned with 0.025 M NaF: The glucose utilization was studied in 9 experiments lasting from 1½ to 4 hours. The results are the same as those with 0.005 M NaF shown in figure 2.

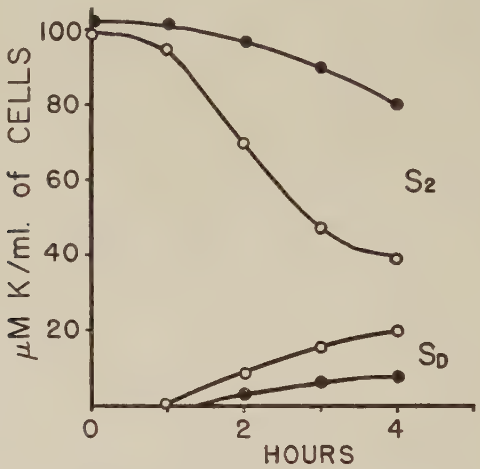

Fig. 5 Comparison of effects of incubation in the presence of 0.025 M NaF alone on cell K with those of incubation with 0.025 M NaF + 0.010 M pyruvate. Coordinates and conditions as in figure 3. Open circles: 0.025 M NaF alone. Closed circles: 0.025 M NaF + 0.010 M pyruvate.

The increase in reducing material (calculated as glucose in fig. 2) in the fluoride poisoned suspension is characteristic. The rates of color development with the Somogyi method showed that the extra reducing material was not glyceraldehyde (Rudney, '49). Paper chromatograms failed to show any sugar except glucose.

All experiments with pyruvate present (26 μM pyruvate per ml of cells) in addition to NaF showed results similar to the curve FP_2 in figure 2. With the technics used, it remains a possibility that the apparent arrest of glucose disappearance represents a balance of the disappearance of glucose and the appearance of other reducing compounds. It is noteworthy that the apparent arrest of glucose consumption occurs at about the same time that the rapidly exchanging K first appears.

Lactate production, studied in 4 two hour experiments corresponds to glucose utilization, but in three of 4 experiments, the lactate production is greater than that expected from the glucose consumption in the flasks containing both fluoride and pyruvate. These 4 experiments are summarized in table 5. Oxygen consumption and CO_2 production measured by the Warburg technic are very small and unaffected by fluoride or pyruvate additions.

TABLE 5

Glucose consumption and lactic acid production in μM/ml cells/hr.

Cl MEDIUM		0.025M F MEDIUM		0.025M F MEDIUM + 0.01M PYRUVATE	
Glucose	Lactate	Glucose	Lactate	Glucose	Lactate
2.5	7.5	0.2	0.3	2.3	8.8
7.1	7.3	1.4	1.7	2.6	5.8
4.7	6.7	0.9	1.4	1.4	5.1
4.8	10.6	0	0	2.1	1.4
		Mean values			
4.8	8.0	0.6	0.8	2.1	5.3

The effect of 0.120 M NaF on K exchange: As noted under methods, NaF replaced NaCl in the medium during washing as well as during incubation so that the ionic strength in these experiments was the same as in the others reported. The cumulative loss of K in an experiment extending 5 hours is shown in figure 6. This loss is about the same as that observed in the presence of 0.005 M NaF in the first two hours of the experiments, as shown in table 1. After 5 hours, however, the K loss is intermediate between that seen with 0.005 M NaF and that seen with 0.025 M NaF. There is no change in cell water. The K^{42} disappearance curves show the presence of a rapidly exchanging fraction of cell K which is much smaller than that found with 0.025 M NaF. There is only a very small effect of pyruvate on K loss (fig. 6) and no clear effect upon K^{42} exchange.

Because K^{42} was not added to identical red cell suspensions at frequent intervals from the beginning of the experiment, the time of appearance of the rapidly exchanging fraction of cells is not known. Even if it is assumed that S_D was present from the beginning of the experiment, it was not measurable until three hours (fig. 6).

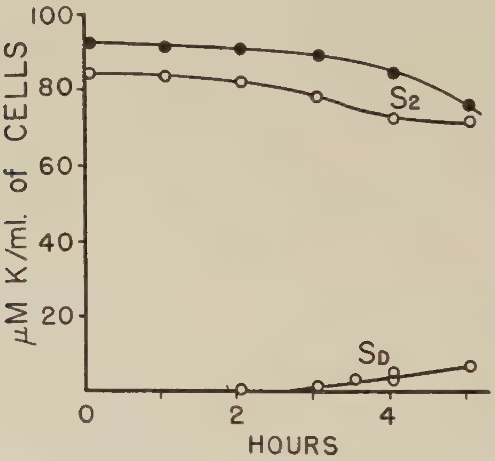

Fig. 6 Comparison of effects of incubation in presence of 0.120 M NaF ± 0.010 M pyruvate. Coordinates and conditions the same as in figure 6. Open circles: 0.120 M NaF alone. Closed circles: 0.120 M NaF + 0.010 M pyruvate. S_D is the same in both suspensions.

Without the knowledge of when cell K first becomes inhomogeneous, the isotope data do not permit the calculation of exchange rates. If it is assumed that S_D is not present for the first hour, and that the K^{42} enters all cells uniformly at first, the entrance rate calculated by equation 4 is 0.3 μM K per ml of cells per hour, and the exit rate is 1.4 μM K per ml of cells per hour. Pyruvate affects these rates little. No experiments on glucose consumption were done.

DISCUSSION[4]

0.005 M NaF: The relationship of the exchange data to energy metabolism is the problem of major interest. It is essential to point out that the arrest of glucose consumption does not arrest all exergonic reactions which might be coupled to K transport. The effect of NaF on glycolysis in red cells was originally described by Dische ('36). The step most sensitive to fluoride is the enolase reaction. When this reaction is inhibited the supply of pyruvate is cut off and the coupling via DPN between the reduction of pyruvate to lactate and the oxidation of triose phosphate to phosphoglycerate is arrested. As a result, no "high energy" phosphate is produced, and that present in the system is used up to phosphorylate glucose, possibly by other transphosphorylations, and by hydrolysis. HDP and triose phosphate do accumulate (Dische, '36). This is presumably the reason for the initial disappearance of glucose in the fluoride poisoned cells shown in figure 2. In addition, the ribose moiety of the nucleotides is known to be converted to HDP in fluoride inhibited hemolysates (Dische, '51). After the first hour of incubation, ester phosphate is hydrolyzed (Mueller and Hastings, '51). This may account for the reappearance of reducing material in the present experiments. Diphosphoglycerate is unaffected (Mueller and Hastings, '51). These reactions are exergonic; they might conceivably be coupled to transport.

On the other hand, in spite of the high consumption of glucose and yield of lactate, the metabolism of glucose and pyruvate in the presence of NaF is much less exergonic than normal glycolysis. The reduction of pyruvate allows the oxidation of triose phosphate to proceed. 2, 3 diphosphoglycerate and lactate are the main products in human cells (Rapoport and Guest, '42). The ATP phosphate used to phosphorylate glucose may be returned to ATP via 1, 3 diphosphoglycerate or accumulate as 2, 3 diphosphoglycerate. Such accumulation would diminish the supply of ATP and the phosphorylation of glucose and may account for the arrest of glucose uptake shown in curve FP_2 of figure 2. No *net* formation of high energy phosphate results in any case. Nevertheless, the net reaction:

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[4] In this discussion, the following abbreviations are used: ATP, HDP and DPN. These stand for: adenosine triphosphate, hexose diphosphate, and diphosphopyridine nucleotide, respectively. The term "high energy" phosphate radical refers to the phosphate groups in ATP, ADP and phosphoglycerate which are linked to the remainder of the molecule by acid anhydride bonds and to the enol phosphate of phosphopyruvate.

Glucose + 2 phosphate + 2 pyruvate ⟶ 2 3-phosphoglycerate + 2 lactate is exergonic. Using the data of Burton and Krebs ('53) for the sum of the reactions involved, at 25°C, pH 7, and 0.010 M concentration of all other reactants and products, the decrease in free energy is 10,000–12,000 calories for the recation above.[5] These facts make interpretation of the exchange data more difficult: the inhibited cell is not inert; and even when glucose use and lactate production are restored by pyruvate, it is not normal.

The K movements observed in this metabolic situation are to be compared with those expected from unrestricted diffusion, using the equation of Ussing ('49a) and Hodgkin and Huxley ('52):

$$\ln \frac{K_{in}}{K_{out}} = \frac{(E - E')F}{RT} \qquad \text{Eq. (6)}$$

For the red cell, E is calculated from the concentration ration of the Cl ion:

$$E = \frac{RT}{F} \ln \frac{[Cl]_o}{[Cl]_i}$$

where the subscripts i and o indicate intracellular and extracellular concentrations. E′ is the concentration cell potential which would be developed by the concentration difference of the ion considered across the cell membrane:

$$E' = \frac{RT}{F} \ln \frac{[K]_i}{[K]_o}$$

F, R, and T have their usual significance.

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[5] This calculation is based on the assumption that all 1, 3 diphosphoglycerate is converted to 3-phosphoglycerate, so that the contribution of the isomerase reaction of Rapoport and Luebering ('50) resulting in the formation of 2,3 diphosphoglycerate is neglected. It is a crude estimate of *in vivo* energetics.

The theoretical flux ratios expected from equation 6 have been calculated for the experiments reported. The results in experiment V117, assuming E = 9.6 mV., and the observed flux ratios for the same experiment are shown in table 6. Early in the experiment, and throughout experiment V91, the ratios are much higher than predicted by equation 6. This means that the rate of K exchange is not determined exclusively by the difference in electro-chemical potential between the K inside and outside the cell; K uptake is higher than predicted. Late in experiments V117 and V96 there is a trend towards agreement. This may mean that the mechanism of K movement is changing. Since there is suggestive evidence in experiment V96 of the formation of a rapidly exchanging fraction of cell K, this could indeed be true. When pyruvate is present, the flux ratios are much higher, and approach closely those observed in normal cells. A spectrum of flux ratios has thus been observed. The ratio is highest and quite constant in the presence of fluoride and pyruvate.

TABLE 6

Comparison of experimental flux ratios in the presence of 0.005M NaF with those predicted by equation 6

Experiment V–117

FLASK	TIME	E — E'	M_1/M_2	M_1/M_2	
		(m V)	(E. 6)	(Eq. 3/Eq. 1)	(Eq. 2/Eq. 1)
F	0	76.3	0.06	0.5	0.4
	1	72.4	0.07	0.4	0.4
	2	68.6	0.08	0.4	0.4
	3	64.9	0.09	0.2	0.3
	4	62.1	0.10	0.1	0.3
	5	58.3	0.11	0.06	0.25
3F	3	68.2	0.08	0.3	0.3
	3.5	66.3	0.08	0.3	0.3
	4	65.7	0.09	0.3	0.3
	4.5	64.5	0.09	0.3	0.2
	5	62.4	0.10	0.3	0.2

In the course of the incubation with fluoride alone, the ratio is intermediate early in the incubation and falls to low values with time.

In all these experiments, the discussion above indicates that the K uptake during 4–6 hours of observation must have occurred without *net* synthesis of high energy phosphate radicals; K uptake coupled to ATP hydrolysis in an irreversible reaction is thus unlikely. The data reveal, however, a delicate relationship between K exchange and the residual metabolism of the cell. Flux ratios higher than those predicted by equation 6 have been encountered in a variety of other inhibited systems (Ussing, '49; Love et al., '55; Tosteson, et al., '55; Hodgkin and Keynes, '55). The concept of exchange diffusion has been given much attention in explaining these ratios because it explains such ratios in the absence of exergonic reactions of the cell. This concept seems less helpful in explaining the current experiments simply because it fails to relate transport to metabolism.

0.025 M and 0.120 M NaF: The effect of 0.025 M NaF has little apparent relation to the effect of NaF at low concentration except early in the incubation. Uptake is more abruptly arrested; the early division of cell K into two fractions is invariable. This is of interest for two reasons. First, the fact that it occurs suggests that there is some obligatory dependence of the normal restricted permeability of the red cell to K upon the intact transport mechanism in this situation. The results of Taylor et al. ('52) for several, but not all of the inhibitors tested, are consistent with this idea. Second, the course of the progressive increase of the rapidly exchanging cell K with time is of interest. The fraction, S_D/S_2 increases with time in an S shaped curve. This suggests the progress of a reaction requiring an induction phase for the formation of an essential intermmediate. This curve also resembles the hemolysis curve of red cells in hypotonic saline, which suggests that it is the individual cells which have a range of susceptibility to the action of NaF. The data themselves provide no information, of course, on whether S_D is a fraction of K in each cell or all the K in a fraction of the cells, but some of the experiments in the following paper (Eckel, '58) suggest the latter.

The effect of 0.120 M NaF has received little attention in this laboratory. Our data show only that its action is qualitatively identical with that of 0.025 M NaF, but that the higher NaF concentration drastically inhibits the formation of a rapidly exchanging K fraction. The mechanism of action of pyruvate in the presence of higher NaF concentrations is not clear.

SUMMARY

1. A convenient and flexible kinetic analysis of K exchange in a closed system has been described. This analysis is useful when inward and outward exchange rates are different and vary with time.

2. The effect of NaF on the K exchange of the human red cell has been examined at three NaF concentrations. The additional effect of pyruvate has been studied in each case.

3. In 0.005 M concentration, NaF gradually slows uptake from 50% to 25% of normal over a 5 hour period. Outgo is unaffected or diminishes less markedly so that net K loss occurs. Cell K may remain homogeneous or divide into two fractions. The addition of pyruvate almost completely abolished the net K loss, and uptake and outgo remain nearly equal at about 70% of normal for 5 hours.

4. In 0.025 M concentration, the initial effect of NaF (for the first ½–1 hour) is identical with that of low NaF concentration. Thereafter cell K divides into two fractions. The first fraction (S_R) decreases with time and exchanges immeasurably slowly with medium K. The second (S_D) increases with time and exchanges almost immeasurably fast with medium K. An extremely rapid loss of cell K parallels the increase in S_D. The addition of pyruvate slows the formation of S_D and diminishes net K loss.

5. In 0.120 M concentration, the effect of NaF is qualitatively identical with that of 0.025 M concentration, but the

size of S_D and the amount of net K loss are markedly diminished. Pyruvate is without effect.

6. Glucose utilization is inhibited in the presence of NaF at any concentration. Lactate production has been found inhibited when this has been studied in the presence of 0.025 M NaF. Pyruvate partially restores glucose consumption in the presence of 0.005 M and 0.025 M NaF. Lactate production from pyruvate is similarly partially restored in the presence of 0.025 M NaF and pyruvate.

7. These results have been discussed as they relate to passive movement of K independent of metabolism, and to active transport of K related to the known reactions of glucolysis.

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POTASSIUM EXCHANGE IN HUMAN ERYTHROCYTES.

II. THE DIVISION OF CELL POTASSIUM INTO TWO FRACTIONS DURING INCUBATION WITH 0.025 M NaF

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EIGHT FIGURES

INTRODUCTION

Human red cells incubated in the presence of 0.025 M NaF lose K at a rapid rate (Wilbrandt, '37, '40). Isotopic studies described in the previous paper (Eckel, '58) indicate that the process of K loss is accompanied by the division of red cell K into two fractions. The first exchanges almost immeasurably fast with the K in the extracellular fluid (ECF). The second apparently does not exchange with ECF K.

The studies reported in this paper are concerned principally with the behavior of the rapidly exchanging fraction of cell K. An estimate of K exchange prior to the time of appearance of this fraction is made. The time at which this fraction first appears has been measured. The total amount of cell K, and the amount of rapidly exchanging cell K have been measured as functions of the time of incubation, and of the ECF K concentration. Using the technic of differential osmotic hemolysis, it has been possible to show that the rapidly exchanging K represents all the K in a fraction of the cells, rather than a fraction of the K in all the cells, at least early in the incubation. An attempt has been made to measure the rate of

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K exchange between the ECF and the rapidly exchanging cells. Finally, a few metabolic studies on these cells are reported.

METHODS

The methods are the same as those described in the preceding paper (Eckel, '58) with a few additions. In experiments during which samples of the medium were obtained every 4–8 minutes, hematocrit measurements were made every 30 minutes and the hematocrit calculated by interpolation for the time of each sample. In the experiments in which NaCl or KCl was replaced by mannitol, the final concentration of mannitol was 0.023 gm per 100 ml of suspension medium for each mM per liter alkali metal replaced. This amount proved to be iso-osmotic with NaCl in preliminary experiments. The nucleotides, adenosine triphosphate and adenosine diphosphate (ATP and ADP, respectively) were measured by their ultraviolet absorption after isolation on a Dowex 1 column as described by Cohn and Carter ('50). The osmotic hemolysis tests were performed as described by Shen et al. ('43).

The terminology, the kinetic analysis giving the inward and outward exchange rates, and the method of determining the rapidly and slowly exchanging fractions of cell K (S_D and S_R, respectively) have been presented in the accompanying paper. In the experiments presented below, the exchange rate between S_1 and S_D is estimated approximately. This requires knowledge of the total amount of K in the exchanging system, which is estimated in separate flasks by means of isotope dilution, using equation 5 of the previous paper. After three hours, R/a_1 (that is, $S_1 + S_D$) becomes quite constant; thus S_1 and S_D are now a system containing a constant amount of K. It is then possible to estimate the exchange rates by use of equations 1 and 2 of the previous paper: R/a_1 is substituted for S, and S_D is substituted for S_2. This is permissible because the second fraction of cell K, S_R, fails to exchange measurably with S_1, and is thus excluded from the calculation. One and a half hours after the start of the experiment, however, the total K involved in the exchange process (R/a_1) is rapidly increasing, and the kinetic analysis of equations 1 and 2 can not be applied since they are based on constant K in the exchanging system. The inward rate, ρ_1, can be measured, however, by means of equation 4 of that paper when K^{42} is added at this time and ρ_2 found by means of equation 3. Two processes contribute to the initial disappearance of K^{42} from the medium at that instant of time. The first is increasing dilution of K^{42} in the expanding S_D. This can be determined from the rate of disappearance of K^{42} from a flask to which K^{42} was added at zero time. This is subtracted from the initial rate of disappearance of K^{42} added at 1½ hrs. to yield an estimate of the second process: the rate of entrance of radioactivity into the rapidly exchanging fraction of cell K present at the moment of addition of K^{42} which is used to calculate ρ_1.

RESULTS

The time of appearance of S_D: The time at which a rapidly exchanging fraction of cell K first appears was studied in two experiments. The more detailed is presented. K^{42} was added at 0, ½ and 1 hour to three flasks which were made 0.025 M with respect to fluoride. Six ECF samples were obtained from each flask during each ½ hour period. The disappearance of K^{42} is shown in figure 1. The calculated exchange rates are shown in figure 2.

The criterion for the presence of two cell K fractions with differing exchange rates is that used in the previous paper. When the calculated rates of exchange (assuming all cell K is exchanging) are significantly higher immediately after addition of K^{42} to one flask than in another flask in which K^{42} has been exchanging for a period of time, a rapidly exchanging fraction is present. The data in figure 2 indicate that there is no evidence of two fractions of cell K at 30

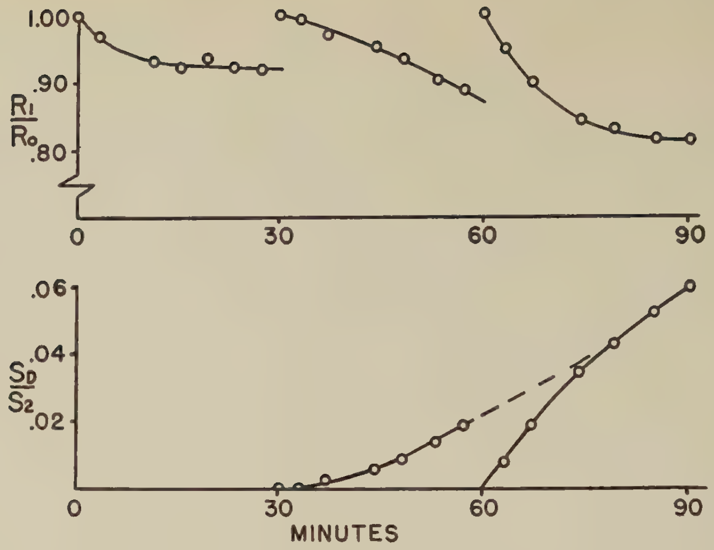

Fig. 1 Upper half: Fraction of K^{42} remaining in the ECF of red cell suspensions incubated at 38°C in the presence of 0.025 M NaF (ordinate) as a function of time of incubation (abscissa). The three curves represent three identical suspensions to which K^{42} was added at 0, 30 and 60 minutes, respectively.

Lower half: Fraction of cell K present as S_D (ordinate) as a function of time of incubation (abscissa).

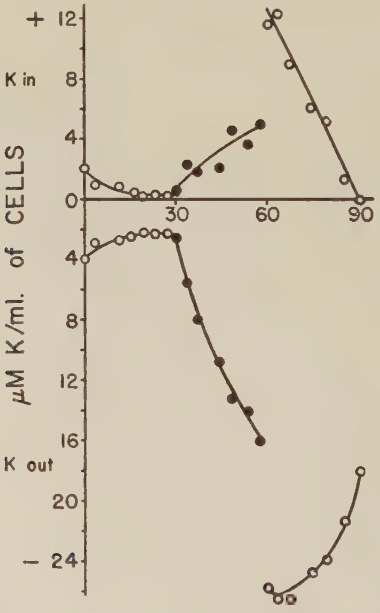

Fig. 2 Apparent values of K_{in} (above abscissa) and K_{out} (below abscissa) in µM K/ml cells/hr. plotted on the ordinate as a function of time (abscissa). Alternate solid and open circles represent separate identical flasks. K^{42} data from figure 1.

minutes, but that at 60 minutes, two fractions do exist.[2] The size of the more rapidly exchanging fraction and its rate of exchange with the K of the medium cannot be determined from the data. Two assumptions are made to determine its size. First, the rapidly exchanging fraction is assumed to be the same K fraction demonstrated as S_D later. Its rate of exchange with the K in the medium is assumed to be as rapid. The fact (see below) that the rate constant for K entrance is quite constant between 1½ and 3 hours makes this plausible. Second, K^{42} entering the remainder of cell K is negligible. This appears likely from the curve R_1/R_0 before S_D is formed (fig. 2, 10–30 min) and from the observation later in such incubations that after equilibration with S_D, little K^{42} enters the cells (e.g., fig. 7). With these assumptions, the size of S_D is approximated according to equation 5 of the accompanying paper.

While the abrupt discontinuity in exchange rates noted between flask 2 and flask 3 at one hour establishes the existence of S_D at this time, the first evidence of its occurrence is between 30 and 33 minutes. The initial rates of flask 2 are the same as those observed in flask 1, indicating that K^{42} is exchanging very slowly, and presumably with all cell K. The rapid increases in rate subsequent to that time in flask 2 strongly suggest rapid equilibration with a nascent rapidly exchanging cell fraction (S_D). In the lower half of figure 1 is plotted the estimated size of S_D as per cent of cell K. The points are calculated assuming that K^{42} entrance is into S_D and that it equilibrates so rapidly with this very small pool that K^{42} entrance does indeed measure the appearance of S_D.

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[2] The falling values for both K_{in} and K_{out} between one and 1½ hours shown in figure 2 are a consequence of the assumption used in calculating the data. This assumption is that K^{42} is exchanging with all of cell K. When the total K actually involved in the exchange process (in this case $S_1 + S_D$) is significantly smaller than the value used in calculating the results (in this case S_2), the calculated rate at which isotopic equilibrium is reached $[d \ln(R - a_1S)/dt]$ is spuriously lowered as the experiment proceeds. (The same artifact can be demonstrated with the data shown as open circles in fig. 7.) The lower half of figure 1, and figure 2 thus represent two quite different interpretations of the same isotope data. The more nearly correct (fig. 1) is chosen on the basis of other information which supplements this experiment.

Total cell K(S_2) and rapidly exchanging cell K(S_D) as functions of time of incubation and ECF K concentration: In the presence of 0.025 M NaF, the extent of the K loss from cells appears to be a function of the time of incubation (Eckel, '58, fig. 3). Because the K lost from the cells accumulates in the medium, ECF K concentration also increases with time, however. The following experiments were done to determine separately the significance of these two variables.

Four aliquots of a red cell suspension were incubated in the presense of 0.025 M NaF, but with varying initial ECF K concentrations. K^{42} was added at zero time, and at three hours the hematocrit and the distribution of K in the system were studied (fig. 3). The total amount of cell K varies

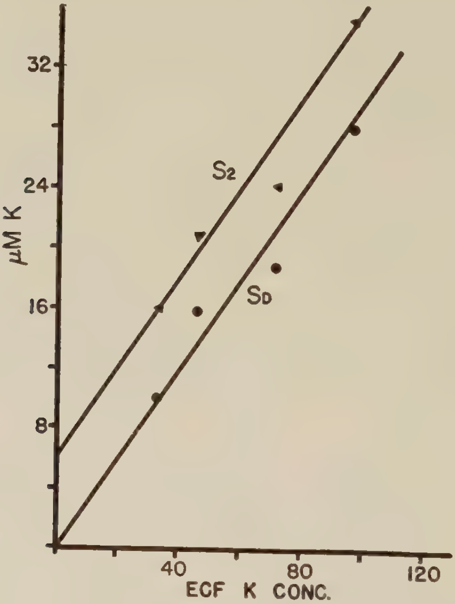

Fig. 3 S_2 and S_D in μM K in the cells of 1 ml of red cell suspension (ordinate) after three hours incubation at 38°C with 0.025 M NaF, plotted against ECF K concentration in mM/l (abscissa). The hematocrits at three hours were .284, .317, .319, .380 for the 4 suspensions in the order of ascending ECF K concentration.

directly with the ECF K concentration. The variable fraction of cell K is $S_D:S_R$ shows no dependence on the ECF K concentration. The hematocrit data in the legend of figure 3 indicate that cell water is also a function of the K concentration in the medium. The same dependence of the amount of K in S_D (and consequently in S_2) was shown in two of three experiments performed in the same manner after 1½ hours incubation with 0.025 M NaF, at the time S_D first becomes clearly evident. The failure in one experiment is ascribed to the limitations of the technic when S_D is small compared to R/a_1 and S_1.

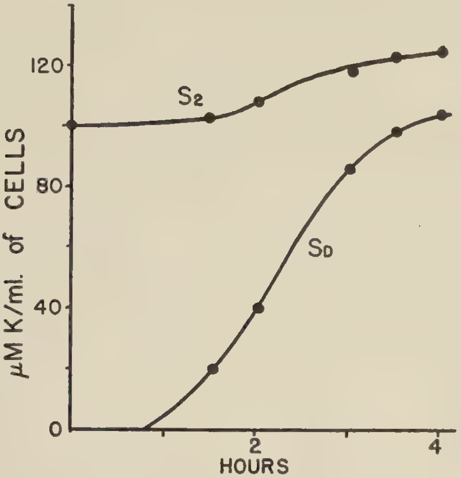

Fig. 4 Total cell K (S_2) and rapidly exchanging cell K (S_D) (ordinate) plotted against time of incubation with 0.025 M NaF (abscissa). ECF K constant at 130 mM/l. Initial hematocrit, .382; subsequent hematocrit values in chronological order: .388, .405, .435, .452, .456.

In experiments where the ECF K concentration is initially 130 mM/l, it remains unchanged during the experiment. The effect of time of incubation with NaF can thus be studied independent of the K concentration in the medium, as shown in figure 4. The cells actually gain K and water and the increases are coincident with the rapid increase in S_D.

S_D as a function of ECF Na concentration: In the experiments described above, Na concentration varied inversely with K in the ECF, so that ionic strength remained constant. It was conceivable that the size of S_D was an inverse function of the ECF Na concentration, and not a direct function of the ECF K concentration. Varying amounts of NaCl were replaced with mannitol in the medium. Two experiments in which S_D was measured at 1½ hours failed to show a clear dependence of S_D upon ECF K or (ECF Na) just as was noted above in an electrolyte medium. The size of S_D after two hours in the mannitol media is shown in table 1 as a function of ECF K concentration in two experiments. The dependence of the size of S_D on the K concentration of the medium and its independence of the ECF Na concentration is evident. In these experiments, no relation is seen between the change in cell water and the cation concentrations of the medium.

TABLE 1

Relation of S_D to ECF concentration of Na and K after two hours incubation in 0.025M NaF with varying amounts of mannitol

Exp't	ECF K	ECF Na	S_D µM/ml cells	Hct.$_0$	Hct.$_{2\ hrs.}$
IV 72	13.4	75.8	4.8	.356	.349
	16.2	68.1	9.8	.410	.376
	33.9	144.1	13.0	.381	.337
	36.4	49.4	13.7	.386	.343
IV 67	13.4	51.8	5.9	.332	.329
	18.2	58.5	12.3	.373	.340
	22.9	151.2	16.0	.369	.351
	35.4	53.8	18.7	.330	.297

The localization of S_D: Does the rapidly exchanging K (S_D) represent a fraction of the K in each cell which has a different role from the remainder, or does S_D represent a fraction of the total cell population in which K behaves differently from the K in the remainder of the cells? To answer this question the osmotic fragility of cells incubated 1½ hours and three hours with and without 0.025 M NaF was examined. The hemoglobin and the K in the supernatant fluid were measured. The results are shown in figure 5 for cells incubated 1½ hours and in figure 6 for the same cells incubated three hours. The lower parts of each figure give a picture of the range of susceptibility of each type of cell to K loss and to hemoglobin loss or lysis as a function of the tonicity of the medium.

The normal distribution of the susceptibility of control cells to osmotic hemolysis is apparent from the middle parts

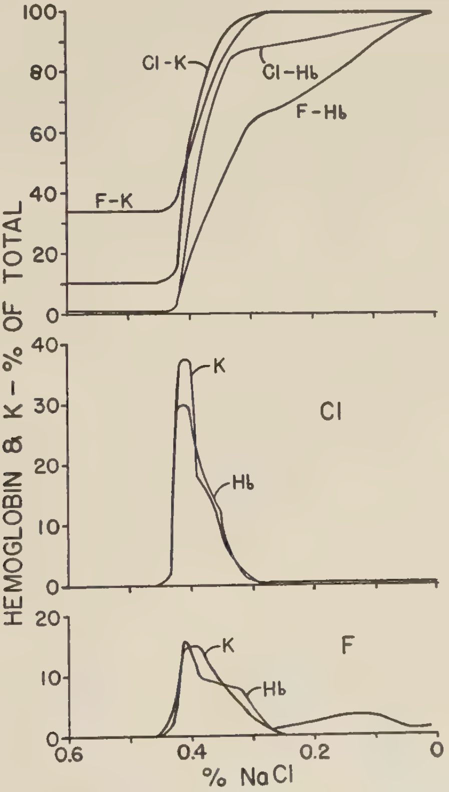

Fig. 5 Per cent of total Hb and K in 1 ml of red cell suspension incubated 1½ hours with 0.025 M NaF at 38° appearing in hemolysate (ordinate) against tonicity of saline used to cause hemolysis. Upper half: observed data. Middle and lower thirds: first derivative of curves in upper third, for control cells (Cl) and NaF cells (F).

of figures 5 and 6. In addition, these data show that K loss and lysis are inseparable in normal cells. Incubation with NaF leads to the appearance of cells resistant to osmotic hemolysis. These increase with time, as does S_D. The fraction of cells resistant to osmotic hemolysis corresponds approximately with the fraction of cell K in S_D. This comparison is made most appropriately with the experiment shown in figure 4: when the K concentration in the medium is adjusted so that cell K changes little, the per cent of rapidly exchanging K in the total cell K should then equal the per cent of cells containing S_D in the total cell population. S_D constitutes 19% of cell K at 1½ hours and 73% of cell K at three hours in

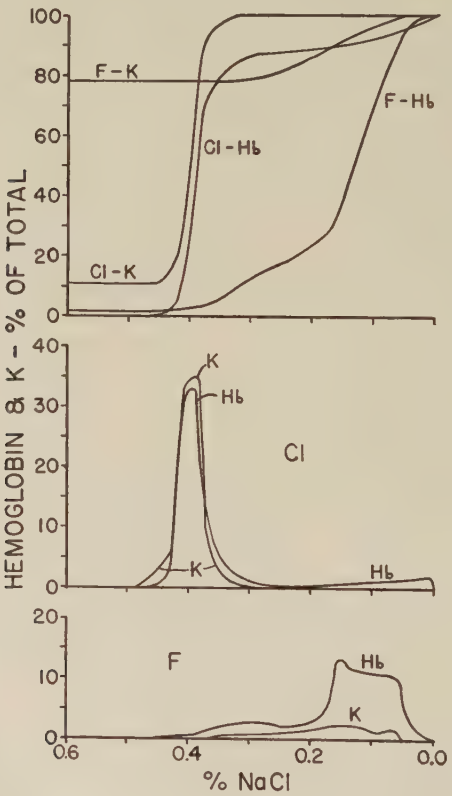

Fig. 6 Same as figure 5, but data collected after three hours incubation.

this experiment. Figure 5 shows that the resistant cells compose 30% of the cell population at 1½ hours; this fraction varies between 16 and 30% in other experiments. After three hours incubation, 83% of the cells are resistant to osmotic hemolysis.

The lysis of the osmotically normal cells after 1½ hours incubation with NaF releases about the same amount of K into the supernatant fluids as does the lysis of control cells. The K in the resistant cells at this time behaves like the rapidly exchanging K of S_D: it rapidly reaches diffusion equilibrium with the K free hemolyzing medium so that the hemolysis of these cells is attended by no increase in the K concentration of the hemolysate. After three hours incubation with NaF, however, the K is only slightly more concentrated with respect to hemoglobin in the osmotically normal cells than in the abnormal and osmotically abnormal cells fail to show the pre-lytic loss of K which would be expected if they contain rapidly exchanging K. The data, then, furnish only suggestive evidence that S_D is confined to a fraction of the cell population. This concept is retained tentatively during the remainder of this paper, however, as the simplest explanation of the localization of S_D.

The rate of K exchange between the ECF and S_D: The actual rate of K exchange between the ECF and S_D is too rapid to define precisely by the technics used. An attempt was made to evaluate the exchange rate at 1½ hours and at three hours in three experiments. Two were performed with an initial K concentration in the medium of 5 mM/l. The shifts of water, radioactivity and K in the first two experiments are similar to those reported in the accompanying paper and are not shown. The third experiment was performed with an ECF K concentration of 130 mM/l. The data on total cell K and the rapidly exchanging cell K of this last experiment are shown in figure 4. The isotope data are presented in figure 7. An estimate of the rates of K movement for the three experiments at 1½ hours is shown in table 2. The graphs on ln $(R - a_1 [S_1 + S_D])$ against time between three and 4 hours for two of the experiments are shown in figure 8; the calculated rates are shown in table 3. The data suggest that S_D is a single fraction of cell K with a single constant exchange rate for K rather than a mixture of many fractions with a spectrum of exchange rates. If the latter were true, one would expect later samples in these experiments to define a smaller slope for the curves in figure 8.

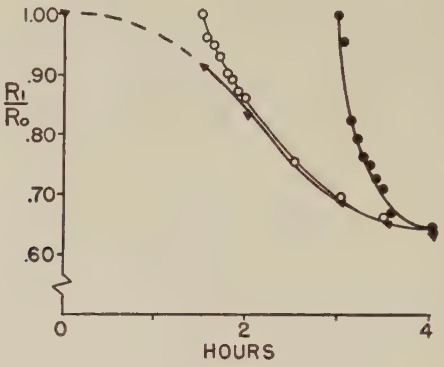

Fig. 7 K^{42} disappearance from the ECF of red cell suspensions incubated in a medium containing 130 mM K/ml at 38 degrees C in the presence of 0.025 M NaF. Ordinate and abscissa as in upper half of fig. 1. Triangles: K^{42} added at 0 time. Open circles: K^{42} added at 1½ hours. Closed circles: K^{42} added at 3 hours. Data from experiment V51, fig. 4. Results are shown in tables 2 and 3.

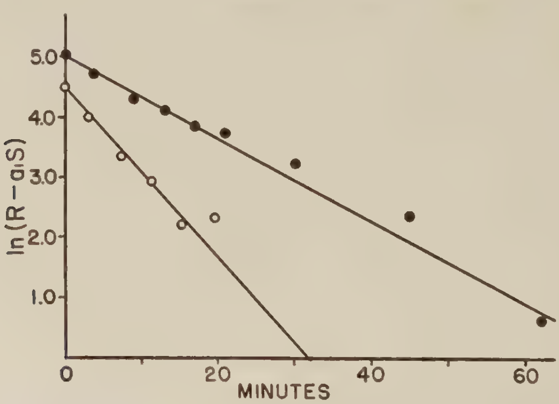

Fig. 8 The function ln $(R - a_1 [S_1 + S_D])$ (ordinate) plotted against time after addition of isotope at 3 hours (abscissa). Derived data from experiment V34 (open circles) and from experiment V51 (figs. 4 and 7) (closed circles).

In both table 2 and table 3 uptake and outgo are presented as though they occurred exclusively between the medium and S_D. This must, of course, be regarded as a first approximation. This approximation is used in the remainder of this section. The validity of this interpretation of the isotope data is considered in the light of all the experimental data in the discussion.

The results, expressed per ml of red cells, indicate very rapid rates of K_{in} and K_{out} at both 1½ and three hours. These rates may be compared with those of the normal cells by expression of these rates per ml of cells containing S_D. The fraction of cells which contain S_D has been estimated from the fraction S_D/S_2 at 1½ and at three hours found in the experiment shown in figure 4. The calculation is as follows:

$$\frac{K_{in}}{S_D/S_2} = K_{in\,D}$$

where K_{in} is the rate of K uptake per ml of cells per hour, S_D/S_2 is 0.192 at 1½ hours and 0.734 at three hours, and K_{inD} is the rate of uptake per ml of cells containing only rapidly exchanging K. This alternative expression of the results seems the most intelligible, since it is an effort to express the exchange for the same area of cell membrane as is available for K exchange in the normal cell. The data in tables 2 and 3 indicate that the absolute rates of K movement in the out of the cell are 60–200 times faster in the damaged cells than in the normal.

If K_{inD} is divided by the K concentration in the medium, a rate constant, k_{in} is obtained which is also shown in tables 2 and 3. This is of interest because if it be assumed that K entrance is a first order reaction, the small changes in this value between 1½ and three hours suggest that the membrane damaged at 1½ hours is not further damaged after the initial insult.

The effect of the K concentration in the medium on the exchange rates is too scanty to permit detailed analysis. At three hours, uptake is dependent, but not exactly proportional to the K concentration in the medium. The day to day variation in these experiments makes it unwise to draw conclusions from comparison of the data obtained on different days, however.

Certain aspects of the apparent K equilibrium between cells and ECF: It is pointed out in the accompanying paper that the net movement of K from cells to medium is small after three hours. The near arrest of K loss occurs when K is

TABLE 2

K exchange rates between S_1 and S_D after 1½ hours incubation in 0.025M NaF

EXPERIMENT	V 22	V 34	V 51
[K] in medium	14.2	12.6	130
S_D/ml cells	8.6	9.3	19.6
K_{in}	27	39	25
K_{inD}	141	203	130
K_{out}	49	62	35
K_{outD}	255	323	182
Net loss	—22	—23	+10
Net loss (per ml S_D)	115	120	57
k_{in}	10	16	1

Rates were calculated using equations 3 and 4.

TABLE 3

Exchange rates between S_1 and S_D after three hours incubation in 0.025M NaF

EXPERIMENT	V 22		V 34			V 51
EQUATIONS USED [1]	1, 3	3, 4	1, 3	3, 4	1, 3	3, 4
K in medium		30.2		29.7		129
S_D/ml cells at 3 hours		23.8		29.0		91.2
K_{in}	155	148	153	130	273	278
K_{inD}	211	202	209	177	372	379
K_{out}	167	159	162	139	267	272
K_{outD}	228	217	221	190	364	371
Net loss	—11	—11	—9	—9	+6	+6
Net loss (per ml S_D)	—17	—15	—12	—13	+8	+8
k_{in}		7		6		3

[1] Equations given in the accompanying paper (Eckel, '58).

still unequally distributed between cells and ECF. It was therefore of interest to determine whether the cell and the medium were in thermodynamic (Donnan) equilibrium with respect to K during the latter part of the experiments. Rapoport and Guest ('39) have shown that the cell: medium concentration ratio of Cl and HCO_3 ions are identical and equal to the reciprocal of the H ion ratio as predicted by the Donnan equilibrium. Harris and Maizels ('52) have confirmed and extended the observations of the Cl and H ion ratios. Accordingly, in 8 experiments, the Cl and K ratios have been compared. These are interpreted assuming that Cl is in Donnan equilibrium across the red cell membrane. In general, both $[K]_i/[K]_o$ and $[Cl]_o/[Cl]_i$ fall as the time of incubation increases, as has been observed by Dunker and Passow ('50) in fluoride poisoned cells. The K ratios in the present experiments are much lower than those found by Harris and Maizels ('52) for cold-stored red cells in which Na had nearly reached Donnan equilibrium and also much lower than those found by Ponder ('49) for fluoride poisoned or control cells incubated for long periods.

The extent of the departure of the K in the system from equilibrium at three hours is evaluated from the relationship

$$\Delta F = RT \ln \frac{[K]_i \ [Cl]_i}{[K]_o \ [Cl]_o} \qquad \text{Eq. (7)}$$

which gives the minimum work of uptake per mole of K. The observed flux ratios are compared with those predicted if the movement of K were determined exclusively by the electrochemical potential difference between the K inside and outside the cell, as would be true in the Donnan equilibrium:

$$\frac{K_{in}}{K_{out}} = \frac{[K]_o \ [Cl]_o}{[K]_i \ [Cl]_i} \qquad \text{Eq. (8)}$$

The observed K and Cl ratios and flux ratios are shown in table 4. The calculated ΔF value indicates that the K is much nearer equilibrium than is indicated by the K ratio. This ΔF value is equivalent to a pure concentration gradient (in the absence of an opposing electrical gradient) of 1.25.

The observed flux ratio agrees with that predicted from the electrochemical gradient alone. This is in clear contrast to the behavior of the normal cell or the cell poisoned with low concentrations of NaF.

In the preceding sections, it has been shown that at a given time, the size of S_D is dependent upon K concentration in the medium and that this K exchanges very rapidly with the K in the medium. Evidence suggesting that S_D is confined to a fraction of the cells has been presented. For the whole cell, at a time when S_D constitutes most of cell K, the ratios $[K]_o/[K]_i$ and K_{in}/K_{out} are close to those predicted from the Donnan relationship. These findings clearly establish that a change in true permeability has occurred in these cells. The experiments described below were directed at the mechanism of formation of S_D, a quite different problem.

TABLE 4

The mean K and Cl ratios and derived data after 3 hours incubation from 8 experiments

TIME	$[K]_i/[K]_o$	$[Cl]_i/[Cl]_o$	ΔF [1]	K_{in}/K_{out} [2] predicted	K_{in}/K_{out} [3] observed
3 hrs.	1.78	.69	+126 cal.	0.8	0.9

[1] Eq. 7.

[2] Eq. 8.

[3] K_{in}/K_{out} observed calculated from experiments V22 and V34, Table 3.

The effect of temperature upon S_D and its K exchange: In experiments lasting 1½ hours, K loss from fluoride-poisoned cells is insignificant at 0°C and very small at 25°C. If fluoride-poisoned cells are incubated at 38° for 30 minutes, or 50 minutes, and then placed at 0°, no further K loss occurs: From these experiments it was suspected first, that the cell changes resulting in the formation of rapidly exchanging cells did not occur at 0° and occurred only very slowly at 25° and second that once initiated at 38°, they did not progress if the cell was kept at 0°. These ideas were confirmed in K^{42} studies. The increase in size of S_D between 60 and 90 minutes was measured in two experiments. In each experiment three flasks of fluoride-poisoned cells were incubated at 38° for 50 minutes before being placed at the indicated temperature. In the first experiment, the flasks were placed at 38°, 30° and 0° at this time and in the second, the flasks were placed at 38°, 25° and 0°. In each case 10 minutes for equilibration at the new temperature was allowed and the experiment continued for an additional half hour until 1½ from the beginning of the experiment. In the last half hour S_D increased 2.7 fold per 10° rise in temperature between 30° and 38° and 2.2 fold per 10° rise in temperature between 25° and 38°. The Q_{10} between 0° and 25° was 1.2; that between 0° and 30° was 0.6. The process of membrane change which permits rapid K exchange requires moderately high temperatures (25°) for its occurrence.

The process of K exchange between S_D and the ECF is much less sensitive to temperature. Two experiments were done after three hours incubation at 38° in fluoride containing media. At this time, S_D is large and changing slowly. The initial disappearance of K^{42} at this time is a good measure of the inward exchange rate. In each experiment, one flask was placed at 30°C at two hours 50 minutes and the other flask left at 38°C. K^{42} was added to both flasks at three hours. The inward exchange rate showed a Q_{10} of 1 in one case and 1.5 in the second experiment. In each case, ECF K concentrations were the same. The higher Q_{10} is equivalent to an activation energy of 4,800 calories.

Metabolic studies related to K exchange: Adenine nucleotides were measured before and after incubation in control and fluoride containing media with and without pyruvate in 6 experiments. The results before and after two hours incubation for one of these are shown in table 5. The phosphorus metabolism of washed red cells in the basal medium is not maintained in a steady state during incubation, even though K transport remains in a steady state over much longer periods of time. This has been observed by Sheppard and Stewart ('42). The addition of fluoride is accompanied by further loss of "high energy" phosphate. Addition of pyruvate to the fluoride-poisoned cells results in levels of the measured adenine nucleotides which are intermediate between those of the control suspension and the fluoride-poisoned cells at two hours.

Other cofactors of glucolysis were investigated briefly. Paper chromatograms of red cell extracts done according to the technic of Cohn and Carter ('50) were tested qualitatively for diphosphopyridine nucleotide (DPN) by streaking with NaCH and observing the fluorescence under UV light (Kaplan, et al., '51). There was no striking difference between control and NaF poisoned cells. Glutathione was measured by technic of Paterson and Lazarow ('55) without demonstrating gross change in the glutathione content of NaF poisoned cells.

TABLE 5

Nucleotide content of red cells: the effect of fluoride and pyruvate

	INITIAL	AFTER 2 HOUR INCUBATION		
		Cl	F	F+ pyruvate
ADP[1]	0.13	0.12	0.07	0.12
ATP	0.51	0.18	0.03	0.04
Labile P[2]	1.15	0.48	0.13	0.20

[1] Values are in μM/ml of red cells.

[2] "Labile P" is the sum of the two terminal phosphate groups of ATP and the terminal phosphate group of ADP.

In single experiments, the effect of other compounds upon the loss K by washed red cells was tested during the first hour of incubation. Removal of substrate (glucose) diminishes markedly the loss of K due to fluoride. In the presence of glucose, nicotinamide, chosen because of its action in inhibiting the triose dehydrogenase reaction (Feigelson, et al., '51; Alivisatos and Denstedt, '52), as well as iodoacetate (as described by Wilbrandt and others) proved to be as effective as fluoride in causing K loss. The effects of nicotinamide and fluoride on net K loss were additive. K^{42} studies of one hour duration indicate that K^{42} exchanges rapidly in the presence of nicotinamide (2%) just as it does in the presence of

0.025 M NaF. The effect of nicotinamide and iodoacetate on K exchange are shown in table 6.

Wilbrandt's ('40) studies using other compounds to modify the effect of NaF were repeated and several other compounds tested in single preliminary experiments. His results were in almost every case confirmed; our results are not worthy of detailed consideration at this time.

TABLE 6

K exchange of human red cell suspensions in the presence of other metabolic inhibitors at the indicated final concentration during the first hour of incubation at 38°C

	K_{in} [1]	K_{out} [1]	NET LOSS [1]
0.1% Nicotinamide	1.2	3.3	2.1
2.0% Nicotinamide	3.6	11.8	8.2
0.025M Iodoacetate	1.4	7.2	5.8

[1] K_{in}, K_{out}, and net loss calculated according to equations 4, 3, and from ΔS_1, respectively.

DISCUSSION

The three compartment system: The evidence for a cellular localization of S_D at 1½ hours is acceptable. It demonstrates that a constant cellular insult, in this case chemical, may produce changes which might appear progressive in all cells, but which are better described as the successive involvement of one cell after another.

At three hours, the data are inadequate to prove that S_D is confined to a fraction of the cells. Complementary K^{42} data indicating that the lysis of the osmotically normal cells (presumably S_R) releases K of low specific activity into the hemolysate, while the lysis of the resistant cells has no further effect upon the specific activity of the K in the hemolysate are necessary. The possibility exists that the cytological difference between S_R and S_D may disappear between 1½ and three hours and the interpretation given the hemolysis data may then be irrelevant.

The exchange of K between the medium and S_R: In these experiments, the exchange of K between the medium and S_R

can only be inferred from other data because the kinetic analysis is not adequate to demonstrate the exchanges. S_R may lose K by two processes. First, this K pool is converted to S_D as the cell membranes are altered in the course of the incubation. This occurs whether there is net loss or gain of K by the entire cell population. Second, the hemolysis experiments suggest K loss from the cells of S_R into the medium. Since the difference in electrochemical potential of K between S_R and S_1 is probably never even as large as in the normal cell, the occurrence of appreciable net loss would involve some K uptake (and therefore K^{42} uptake) in the reverse reaction. This would be true even if the loss were entirely by diffusion, as is evident from the application of equation 8 to this situation. K exchange between the medium and S_R thus seems probable.

The cell volume changes: The occurrence of cells resistant to osmotic hemolysis which are permeable to cations requires comment. If the cation permeability were restricted to K (or if the cell were merely significantly more permeable to K than to Na), the volume of the cell should be proportional to the K concentration of an isotonic medium of Na and K salts at any given time, as has been pointed out by Boyle and Conway ('41) in a general discussion of this problem. This has been observed in the experiments reported, as indicated by the hematocrit data reported in the legends of figures 3 and 4. In most experiments, however, the number of cells composing S_D (those with the postulated impermeability to Na and known marked permeability to K) increase at the same time the K concentration in the medium is increasing. This situation is too complex to discuss with the data at hand.

Wilbrandt ('40) has observed that the osmotic hemolysis of fluoride-poisoned cells occurs at much higher concentrations of KCL than of NaCl. This too would be expected if these cells were much more permeable to K than to Na.

The metabolic studies: No cofactor of glucoclysis which might be essential to the structure of the membrane cation barrier was found depleted except ATP. Since a separate fraction of red cell ATP has been isolated from the red cell ghost (Prankerd and Altman, '54), it is possible that this densely charged anion has a specific role in the membrane. On the other hand, an alternate pathway of sugar metabolism such as that outlined by Dische ('51) may become operative when ATP falls below an effective concentration. As Wilbrandt points out some intermediate of this scheme, normally present in the membrane might be consumed, or an intermediate itself damaging to the membrane might accumulate.

The high temperature coefficient of S_D formation tends to exclude certain possible actions of NaF. Thus it seems unlikely that fluoride acts by precipitating calcium or magnesium, or forming a complex fluorophosphate, any of which might physically disrupt the membrane. Such compounds, if formed (or the fluoride itself), must interfere with reactions essential to the maintenance of the membrane. The temperature effect should prove useful experimentally in analyzing the problem further. The effect of pyruvate in diminishing the fluoride effect, and that of iodoacetate in mimicking the fluoride effect, as pointed out by Wilbrandt, also exclude a physical explanation of the effect.

To these observations have been added the observations on the effect of nicotinamide. The known relevant effects of nicotinamide are to competitively inhibit diphosphopyridine nucleotidase (Mann and Quastel, '41; Alivisatos et al., '56), an enzyme demonstrated to be present in the red cell ghost, to lead to the synthesis of nicotinamide riboside in the red cell (Leder and Handler, '51), and to competitively inhibit DPN dependent enzymes (Feigelson et al., '51; Alivisatos and Denstedt, '52). The reaction in the glucolytic scheme most sensitive to iodoacetate is the triose phosphate dehydrogenase reaction, which also requires DPN. Fluoride interferes with the formation of pyruvate from glucose, and since the reduction of pyruvate to lactate is coupled to the re-oxidation of DPN, all three inhibitors draw attention to this oxidative step in glucolysis. It must be emphasized that inhibitors are "blunt tools" for the study of intact cells. It is more correct at present simply to state that the energy metabolism of the cell is essential to the maintenance of the membrane.

CONCLUSION

1. The following is the best tentative description of the action of 0.025 M NaF on the K metabolism of human red cells incubated at 38°C in a saline-phosphate buffer:

The process of K uptake is first inhibited, as it is in the presence of 0.005 M NaF, virtually isolating the ECF K from access to the cell K without significantly affecting the outward movement of K. The ratio of uptake to outgo of K quickly approaches that expected due exclusively to the difference in electrochemical potential between the K in the cell and that in the medium. Shortly after the beginning of the experiment, the isolation of cell K by the cell membrane is destroyed in a progressively increasing number of cells. From the derived rate constant for K uptake into these cells, it is concluded that this damage to the membrane is not progressive after the initial insult. The K in the rapidly exchanging cells (S_D) may be rapidly lost into the ECF, or retained in the cells, depending upon the concentration gradient between the K of S_D and the medium. The significant feature of the behaviour of K in this fraction of the cells is not the direction of its net movement, but the extreme rapidity of the opposing transfer rates. The temperature coefficient of this process is low. Late in the incubation, cell K approaches Donnan equilibrium with the K in the medium. The changes in cell water are those expected if the membrane damage affected the permeability of the cell to K without markedly affecting the permeability to Na. The residual, slowly exchanging cells (S_R) show equivocal evidence of K exchange, but do lose K into the medium. The above description is tentative principally because of uncertainties concerning the cellular localization of S_D at three hours and concerning the behaviour of S_R.

2. Studies of the mechanism of formation of rapidly exchanging cells have shown this process has a high temperature coefficient. The only cofactor of glucolysis found reduced in

the NaF poisoned system is ATP. Nicotinamide and iodoacetate produce similar effects on K exchange.

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ELECTROLYTE MIGRATIONS ACROSS THE WALL OF THE GUINEA PIG GALL BLADDER

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It has been known for many years that the gall bladder is capable of concentrating bile salts and pigments by the absorption of water and simple salts. Associated with this process of absorption is a decrease in pH and a fall in the concentration of bicarbonate of gall bladder bile. As early as 1869 Pflüger noted that the CO_2 liberated by acid was greater in hepatic than gall bladder bile of the dog. In 1916 Quagliariello showed that the pH was greater in hepatic than gall bladder bile in the same species. These observations have been extended to a great variety of animals (Okada, '16; Drury et al., '24; Neilson and Meyer, '21).

The present work is a study of the changes in the ionic composition of gall bladder bile in the guinea pig under varying conditions. An unexpected finding was the observation that bicarbonate was secreted into the lumen of the gall bladder under certain conditions both *in vivo* and *in vitro*.

METHODS

In vivo experiments — Guinea pigs weighing from 500 to 1000 gm were anesthetized with nembutal (25 mg/kgm body weight) injected intraperitoneally. Since anesthesia was relatively light, it was found necessary to infiltrate the abdominal wall with 1% procaine. The abdomen was opened by a midline incision. A small block of wood placed at the mid-portion of the back made the gall bladder and common bile duct more accessible. In one group of experiments hepatic bile, gall bladder bile, and blood were obtained. The cystic duct was ligated, the contents of the gall bladder aspirated directly through the wall with a needle and syringe, and the bile injected immediately into a small beaker of oil (liquid petrolatum). The common bile duct was ligated just above the point where it entered the duodenum and was cannulated with a small polyethylene tube which was held in place with a ligature. The abdomen was closed and the polyethylene cannula brought out through the incision, the tip being placed in a small beaker under oil. The flow of bile was instantaneous and constant and in one case where it was measured flowed at a rate of 0.1 ml per minute. Blood was collected in a heparinized syringe at the end of the experiment by cardiac puncture and injected into a centrifuge tube under oil.

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In a second group of experiments various solutions were introduced into the gall bladder. The cystic duct was cannulated with a small polyethylene tube and held in place with a ligature. In some of these experiments the cystic artery and vein were ligated but this did not appear to affect the results since there was abundant blood supply to the gall bladder from the adjacent surface of the liver. The distal portion of the cannula was brought out through a stab wound in the left side of the abdominal wall. The gall bladder was washed three times with the solution to be studied. This solution was introduced into the gallbladder, the cannula was clamped, and the abdomen closed during the experimental period (1 to 6 hours). At the termination of the experiment the abdomen was opened and the solution removed from the gall bladder with a needle and syringe and injected into a small beaker of oil.

In vitro experiments — Animals were sacrificed by a blow on the head and the abdomen quickly opened. The gall bladder was carefully removed and placed in a Petri dish of isotonic saline at room temperature. The contents of the gall bladder were drained onto filter paper and then the interior was washed with bicarbonate-saline with the aid of a blunt needle and syringe. The gall bladder was everted with a stainless steel probe in the manner previously described for the intestine (Wilson and Wiseman, '54). Bicarbonate- saline was introduced into the everted sac with a blunt needle attached to a syringe and as the needle was withdrawn, the neck of the sac was tied. The filled sac was incubated in a 50 ml Erlenmeyer flask containing 2 ml of bicarbonate-saline solution which had been gassed with 95% O_2 and 5% CO_2. The Erlenmeyer flasks were placed in a shaking incubator for 2 hours at 37°C.

Experimental Solutions — The solutions used were: (1) bicarbonate-saline (Krebs and Henseleit, '32); (2) hepatic bile obtained by the previously described method from the same or different animals; (3) sodium dehydrocholate (decholin, N. F.) 120 mM, phosphate buffer 10 mM, sodium bicarbonate 26 mM; (4) Krebs-Henseleit bicarbonate-saline solution with 0.1M Na_2SO_4 substituted for the 0.15M NaCl (all other additions made as usual); (5) congo red, 37 mM (2.5 gm/100 ml), NaCl 70 mM, and sodium bicarbonate 50 mM. The bicarbonate concentration in the Krebs-Henseleit saline solution was increased in some experiments by substituting sodium bicarbonate for NaCl.

Analytical Procedures — Sodium and potassium were determined with the flame photometer (Perkin and Elmer). Chloride was determined by the method of Schales and Schales ('41). In the *in vitro* experiments bicarbonate was determined in Warburg flasks by tipping 0.5 ml of 5N H_2SO_4 (side arm) into 2.5 ml of solution containing bicarbonate (main compartment) previously gassed with 95% O_2 and 5% CO_2. The CO_2 evolved following the tipping of the acid into the bicarbonate solution was measured manometrically. In the *in vivo* experiments the total CO_2 was determined by the method of Van Slyke and Neill ('24). The pH was determined with the glass electrode at 25° (Beckman model C).

RESULTS

Measurements were made of the pH and the concentrations of the various electrolytes of the hepatic bile, gall bladder bile, and blood of ten individual guinea pigs. Table 1 shows the average values and the standard deviation of the mean. The total CO_2 of both gall bladder and hepatic bile was about three times greater than that present in the blood. The concentration of total CO_2 in the hepatic bile was greater than that in the gall bladder bile in nine out of ten animals. Little difference was found in the pH or CO_2 tension between the two biles. The chloride in the gall bladder bile was less than the chloride in the hepatic bile in eight out of the ten animals, bile chloride being about half that of blood chloride. In all animals the potassium was found to be higher in the gall bladder bile. The blood plasma electrolyte values are comparable to human values. In these experiments gall bladder bile represents the end product of the action of the gall bladder on hepatic bile, but the length of time the bile had been in the gall bladder was unknown. It was assumed that anesthesia had no effect on the electrolyte composition of hepatic bile.

TABLE 1

Ionic content of hepatic and gall bladder bile from the guinea pig

Average values are given for estimations in ten different animals. Numbers in parentheses indicate the standard deviation of the mean.

	GALL BLADDER BILE		HEPATIC BILE		BLOOD	
Total CO_2 (mM)	73.8	(2.7)	85.3	(2.2)	27.9	(1.3)
pH	7.92	(0.02)	7.94	(0.02)	7.56	(0.15)
Chloride (mM)	56.0	(2.6)	61.6	(1.9)	108	(1.8)
Sodium (mM)	156	(2.2)	156	(1.3)	147	(0.7)
Potassium (mM)	5.90	(0.11)	4.61	(0.2)	4.09	(0.17)

In order to confirm the results obtained by the above method a sample of hepatic bile of known electrolyte composition was placed into the gall bladder and allowed to remain for varying lengths of time. Table 2 shows that in the 5 and 6 hour experiments there was a fall in total CO_2 with no appreciable change occurring in the 1 and 3 hour experiments. In all these experiments the chloride concentration fell or did not change, whereas the potassium concentration consistently increased. These results are similar to the results obtained by sampling hepatic and gall bladder bile.

TABLE 2

Ionic changes of hepatic bile placed in the gall bladder in vivo

Hepatic bile from the same or different animals was introduced into the gall bladder of an anesthetized guinea pig.

EXPT. NO.	TIME	SAMPLE	VOLUME	pH	TOTAL CO_2	CHLORIDE	SODIUM	POTASSIUM
	hrs		*ml*		*mM*	*mM*	*mM*	*mM*
1	6	Initial	1.5	8.00	81.7	62.0	151	4.1
		Final	1.3	7.85	78.2	57.0	149	7.7
2	5	Initial	2.0	7.92	85.7	58	150	4.0
		Final	1.6	7.79	80.0	57	154	6.1
3	6	Initial	2.0	7.89	79.0	60	144	5.3
		Final	1.5	7.94	73.6	52	140	7.7
4	3	Initial	1.5	7.95	93.5	53	161	5.3
		Final	1.3	8.10	94.0	41	154	6.9
5	1	Initial	1.5	7.95	81.0	51	151	4.2
		Final	1.0	7.99	81.3	51	166	6.1

To test further the action of the gall bladder, artificial solutions of known electrolyte composition were introduced into the gall bladder. Table 3 shows the rise in potassium and, in most cases, the fall in chloride which was previously observed with bile. However, there was usually a rise in pH and total CO_2 which was unexpected. In every case but one there was an increase in bicarbonate concentration. This one case (no. 9) showed no significant change.

A simplified system was then developed with *in vitro* preparations of guinea pig gall bladder to study these ionic changes. Measurements were made of electrolyte concentration changes on each side of the wall of such *in vitro* preparations. A sac of everted guinea pig gall bladder was incubated with bicarbonate-saline on the mucosal and serosal sides. Table 4 shows one such complete experiment. The concentra-

TABLE 3

Ionic changes in saline placed in the gall bladder in vivo

Bicarbonate-saline introduced into the gall bladder for 1 hour

EXPT. NO.	SAMPLE	VOLUME	pH	TOTAL CO_2	Cl	Na	K
		ml		*mM*	*mM*	*mM*	*mM*
1	Initial	1.5	7.41	26.8	130	159	4.6
	Final	0.9	7.75	37.1	108	157	5.8
2	Initial	1.5	7.45	27.0	130	—	4.6
	Final	0.9	7.68	30.2	123	—	7.7
3	Initial	2.0	7.49	30.4	131	157	2.9
	Final	0.9	7.7	34.9	122	—	7.5
4	Initial	2.0	7.49	30.4	131	157	2.9
	Final	1.6	7.69	41.2	114	158	6.0
5	Initial	1.7	—	39.4	112	152	3.1
	Final	1.3	—	50.3	105	158	3.9
6	Initial	2.0	7.70	38.4	115	151	5.0
	Final	1.8	7.57	41.5	110	148	5.9
7	Initial	2.0	7.81	55.3	97	147	4.9
	Final	1.6	7.81	59.6	99	148	5.9
8	Initial	1.5	—	78.8	75	154	6.2
	Final	1.1	—	85.0	71	151	6.8
9	Initial	2.0	7.90	73.4	80	152	4.7
	Final	1.7	7.95	73.6	81	150	5.5

TABLE 4

Ionic changes across the guinea pig gall bladder in vitro

Sac of everted guinea pig gall bladder incubated in bicarbonate-saline containing 200 mgm glucose/100 ml for 1 hour at 37°C. Final wet weight, 160 mg. Gas phase, 5% CO_2 and 95% O_2.

	VOLUME	BICARBONATE		CHLORIDE		SODIUM		POTASSIUM	
		Conc.	Total Amount	Conc.	Total Amount	Conc.	Total Amount	Conc.	Total Amount
	ml	*mM*	*μM*	*mM*	*μM*	*mM*	*μM*	*mM*	*μM*
Initial mucosal	3.00	25.7	77.1	130	390	149	447	4.17	12.5
Final mucosal	3.07 [1]	27.4	84.0	124	381	151	464	4.77	14.6
Initial serosal	0.92	25.7	23.6	130	120	149	137	4.17	3.83
Final serosal	0.85	19.6	16.7	134	114	156	132	3.18	2.70
Mucosal change	+0.07 [1]		+6.9		—9		+17		+2.1
Serosal change	—0.07		—6.9		—6		— 5		—1.1

[1] Calculated from the three measured volumes, assuming no tissue swelling or evaporation.

tion of bicarbonate rose on the mucosal side and fell on the serosal side. There was a net transport of 6.9 μM of bicarbonate across the wall of the gall bladder. Likewise the concentration of potassium rose on the mucosal side and fell on the serosal side giving a net transport of 1.13 μM. About 1 μM was lost from the tissue during incubation. No clear-cut results were obtained in the case of sodium and chloride. The final serosal volume was measured directly while the mucosal volume was calculated from the change in the serosal volume assuming no tissue swelling. Even if the tissue had doubled its initial water content the error introduced into the calculated final mucosal volume of the experiment in table 4 would be less than 3%. Similar results were obtained in five other experiments with respect to the transport of both bicarbonate and potassium ions from the serosal to the mucosal side.

There is a marked discrepancy between the *in vivo* experiments with bile on one hand and the *in vivo* and *in vitro* experiments with bicarbonate-saline on the other; in the former case the total CO_2 concentration fell in the gall bladder while in the latter case the reverse occurred. The possibility was considered that the non-diffusible bile salts present in bile set up a Donnan effect which resulted in the reduction in the concentration of bicarbonate. Non-diffusible anions were, therefore, added to the saline solutions introduced into the gall bladder *in vivo* to determine whether such solutions were capable of reproducing the ionic changes observed with bile. Table 5 shows that when sulfate ion was substituted for chloride in the bicarbonate-saline both the pH and the concentration of bicarbonate fell. This movement of bicarbonate is in the opposite direction from that observed in the absence of sulfate (see table 3). Similar results were obtained with sodium dehydrocholate substituted for chloride. Three further experiments were carried out with congo red at a concentration of 37 mM. The presence of this dye prevented the rise in bicarbonate observed with saline alone although it did not reverse the effect as did sulfate and dehydrocholate.

TABLE 5

The effect of sulfate and dehydrocholate on total CO_2 concentration and pH in the gall bladder in vivo

Bicarbonate-saline introduced into the gall bladder for 1 hour

ADDITION	EXPT. NO.	VOLUME	pH	TOTAL CO_2
		ml		*mM*
Na_2SO_4	(1) Initial	2.0	7.45	27.4
	Final	1.5	7.20	18.4
	(2) Initial	2.0	7.45	27.4
	Final	1.6	7.12	19.6
	(3) Initial	2.0	7.45	25.6
	Final	1.5	7.20	17.5
Sodium dehydrocholate	(1) Initial	2.0	7.61	31.6
	Final	1.6	7.42	28.7
	(2) Initial	2.0	7.61	31.6
	Final	1.5	7.40	26.6
	(3) Initial	1.7	7.61	26.1
	Final	1.2	7.29	23.6

DISCUSSION

The present experiments show that the guinea pig gall bladder acts upon the bile by reducing the concentration of bicarbonate and chloride and increasing the concentration of potassium. The reduction in bicarbonate concentration in the bile of this animal is not as striking as that found with other species. Thus, Neilson and Meyer ('21) showed that of the ten species of animals studied only the guinea pig had gall bladder bile alkaline to phenolphthalein. The average pH of such bile was given as 8.89 by Krantz et al. ('36). The gall bladder of many species (such as dog and man) can reduce both the pH and bicarbonate concentration well below the corresponding values found in blood.

When bicarbonate-saline solutions, similar in ionic composition to that of blood, were introduced into the gall bladder *in vivo*, the bicarbonate concentration rose. This result was entirely unexpected as it was opposite to that found with bile under the same conditions. This effect, however, was confirmed with *in vitro* preparations which were bathed on each side with an artificial saline containing only inorganic salts. The *in vitro* preparations showed a net transport of bicarbonate from one side of the membrane to the other, an effect difficult to demonstrate clearly *in vivo*. It appears, therefore, that the epithelium of the gall bladder is capable of secreting bicarbonate from the blood into the lumen against a concentration gradient. Such a mechanism does not seem to operate in the dog since bicarbonate-saline solutions introduced into its gall bladder showed no appreciable change in concentration (Ravdin et al., '31).

There are a number of explanations that may be formulated to account for the decrease in concentration of bicarbonate in gall bladder bile. A few of these possibilities are: (1) simple diffusion of bicarbonate from the high concentration in bile to the low concentration in blood; (2) secretion of acid into the lumen of the gall bladder by some type of active process; and (3) a Donnan effect due to non-diffusible anions (bile salts) present in bile.

The first possibility does not seem to be in accord with the available data since in the present experiments the bicarbonate concentration usually rose after instilling saline solutions containing high concentrations of this ion. Also, this explanation would not account for the fall in bicarbonate concentration in the gall bladder bile of some animals (dog and man) below the concentration present in blood.

Acid secretion by the gall bladder epithelium cannot be ruled out in the case of many animals but in the guinea pig saline solutions placed in the gall bladder became alkaline rather than acid.

The presence of a high concentration of non-diffusible anion in bile (bile salt) suggests the possibility of the operation of the Donnan equilibrium. Sobotka ('37) has suggested this mechanism to account for the low concentration of chloride in bile. In support of this hypothesis are the results of the experiments with sulfate and dehydrocholate (table 5). Sulfate is known to diffuse across living membranes such as the intestinal mucosa and the kidney tubules much less rapidly than chloride or bicarbonate and, therefore, would be expected to show a Donnan effect in relation to these two ions. The bile salt dehydrocholate is known to be relatively non-diffusible across the gall bladder wall since all the bile salts are concentrated in this organ against a very large concentration gradient (with respect to blood). These two relatively non-diffusible substances cause a reduction in the bicarbonate concentration of saline solutions placed in the gall bladder as would be predicted from the Donnan equilibrium.

It has already been pointed out that the gall bladder of most other animals can reduce the bicarbonate concentration of bile to a much greater extent than that of the guinea pig. In the gall bladder of the latter species there are probably at least two forces acting on the bicarbonate ion, bicarbonate secretion tending to increase its concentration and the Donnan effect tending to diminish it. In the gall bladder of the dog, however, there appears to be no process of secretion (Ravdin et al., '31) and as a result the Donnan effect would be unopposed.

A consistent finding in this study was the rise in potassium concentration in solutions placed in the gall bladder. This result would be expected by a Donnan effect but other factors must also be involved since the potassium movement across the wall occurs in the *in vitro* experiments in the absence of non-diffusible anions.

SUMMARY

Gall bladder bile of the guinea pig was found to have a lower bicarbonate concentration than hepatic bile. Similarly, when hepatic bile was placed in the gall bladder of an anesthetized animal for 5 to 6 hours the bicarbonate concentration fell. In contrast with these findings, the bicarbonate concentration increased when Krebs-Ringer solution was placed in the gall bladder *in vivo* or when this solution bathed the two sides of the gall bladder *in vitro*. The addition of the relatively non-diffusible anions desoxycholate or sulphate to the

Krebs-Ringer solution led to a fall in bicarbonate concentration when placed in the gall bladder *in vivo*. These observations suggest that although the guinea pig gall bladder is capable of secreting bicarbonate into simple salt solutions this effect is completely reversed by the presence of high concentrations of non-diffusible anions. It is suggested that the presence of non-diffusible bile salts in normal bile sets up a strong Donnan effect which results in the fall of the bicarbonate concentration in gall bladder bile.

A consistent finding in this study was the secretion of potassium ion into the gall bladder containing either bile or salt solution.

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